CHAPTER 1

Introduction

1.1Theories/ Principles and Rationale

Telomere is a specific nucleoprotein structure at the end of every eukaryotic chromosome. It differentiates the linear chromosomes from other DNA double-strand breaks, preventing them from end-to-end fusion and DNA degradation [1]. Telomeric DNA is progressively shortened in each round of cellular division due to the end replication problem [2]. In higher eukaryotes such as vertebrates, telomeric DNA consists of several kilobases of the 6-bp (TTAGGG) tandem repeats [3]. This long telomeric repeats allows a somatic cell to replicate for a number of cycles before its telomeres are shortened to a critical length, which then triggers the cell to enter senescence [4].

Telomere length assessment from leukocytes is now one of the biomarkers for aging. Various studies have shown that people with shorter telomeres in their white blood cells are more likely to develop illnesses like cancer, heart disease and Alzheimer's disease [5]. Stress is also associated with shorter telomeres; and stress relief, exercise, or certain nutrients such as magnesium, manganese, zinc, omega-3 fatty acids, vitamin A, B, C, D, E, folate, CoQ10, polyphenols (resveratrol, epigallocatechin gallate) and curcumin, can decelerate the rate of telomere attrition [6-14]. Studies in mice also showed that extending telomeres via an enhancement of telomerase activity, either by gene therapy or telomerase activator, could lengthen their lives [15, 16]. Moreover, a commercial telomerase activator TA-65® has been claimed to be able to rejuvenate cells through maintaining of telomere lengths [17]. People who took TA-65® reported that they experienced improvement in some keys physical performances [18].

Several techniques have been applied to assess telomere length; some provide results in overall or mean telomere length, others give the length of a specific chromosome. There are growing evidences showing that having a single shortest telomere length is sufficient to trigger cellular senescence [19]. Therefore, the shortest telomere length is a better biomarker for longevity than the average telomere length.

The telomere length of Chromosome 17p (Chr17p) is now believed to be the shortest length among all telomeres [20-22]. It might be more useful to obtain the telomere length from Chr17p for clue of longevity than the overall telomere length. Currently, single telomere length analysis (STELA) is a method of choice when the Chr17p telomere length is measured. However, the method is quite cumbersome and requires radioactive probe for detection. Here we present a non-radioisotopic alternative method to detect Chr17p telomere length. Our method involves 4 steps (Figure 1.1); (i) modification of the 3' telomeric DNA end with a DNA adapter, (ii) primer extension of modified 3' overhang using a RP primer targeted at the modified sequence, (iii) amplification of the extended product by PCR using a Chr17p-specific primer targeted at the subtelomeric sequence, and (iv) detection of amplified products by Southern blotting with digoxiginin-labelled probe, followed by chemiluminescence detection system. This method is simple and can be adopted in most laboratories.

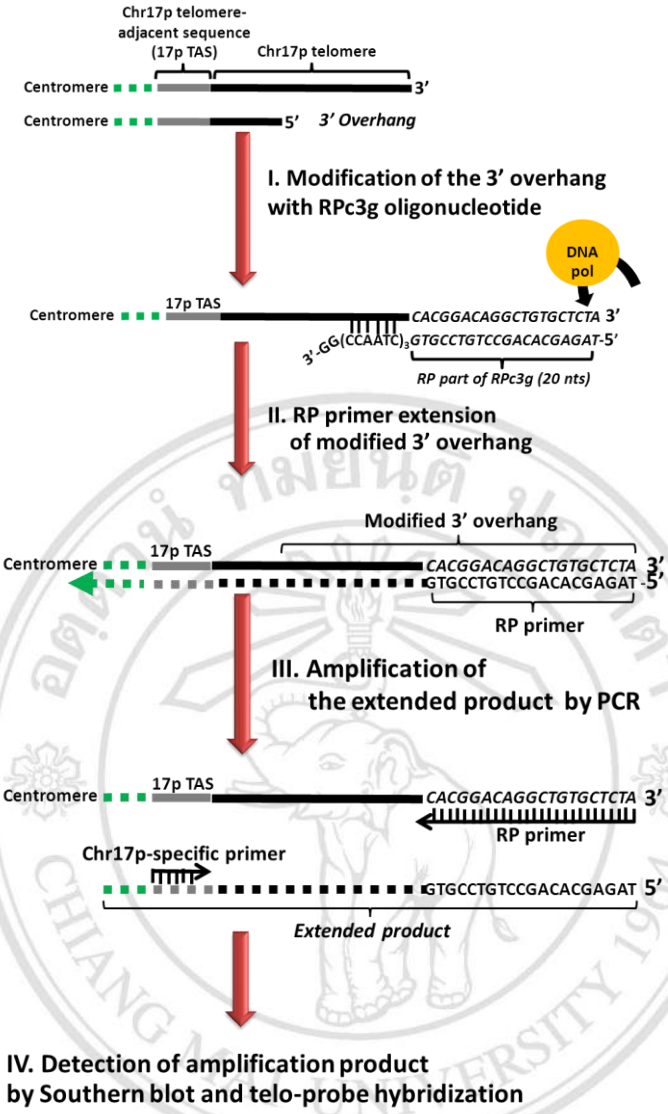

Figure 1.1 The method for measurement of Chr17p telomere length

The 3' telomeric ends are first extended with RPc3g adapter DNA. The 3'-end of this primer anneals to the last 3 repeats of the TTAGGG 3'-overhang of telomeric DNA, with a two GG mismatch bases at the 3'-end to prevent further polymerization of the adapter. The 5'-overhang of this primer is then used as the template to extend the telomeric ends with the RP adapter sequence. Once the telomeric end is extended by the adapter, the Chr17p telomeric DNA can be amplified using RP primer and Chr17p-specific primer (17p6), which anneals specifically to the telomere adjacent sequence (TAS) of Chr17P. The PCR products are then separated by agarose gel electrophoresis and the Chr17p telomeric DNAs were detected by Southern blotting analysis with chemiluminescence detection system.

1.2 Literature reviews

In the late 1930s, Hermann J. Muller and Barbara McClintock defined the natural chromosomal ends as specialized structures called telomeres [23]. Telomeres are protein-DNA complexes that form capping structures to stabilize chromosomal ends and prevent them from being recognized as DNA double-stranded breaks [24]. In addition to physical protection of chromosome ends, eukaryotic telomeres also play an important role in cellular processes including chromatin organization and control of cell proliferation.

1.2.1 Telomere structure

Telomeres consist of repetitive DNA sequences, which are highly conserved during the evolution, and specific protein complexes. Telomere length is species specific; the average length of telomeric tracts in budding yeast is about 300 bps, while mammals usually have much longer telomere length. For example, most mice strains have very long telomeres (20-150 kb), and human telomeres usually extend from 2-30 kb in length. Variability within species might exist, as well as variation due to the nature of the tissues, the age, and the normal or pathological status of the cells. Telomeric DNA sequence is also varied among species; it usually consists of tandem repeats of 6-8 base pairs, in which one strand of the duplex contains a G-rich sequence. This G-rich sequence is extended beyond the duplex region and forms a 3'-overhang. In human, the repetitive DNA sequence is 5'-TTAGGG-3', and the 3' G-rich overhang, extends about 50-300 nucleotides [24-26]. The sub-telomeric sequences, consisting of fairly repetitive DNA, are very little conserved during the evolution. Telomeric sequences do not contain protein-encoding genes but are crucial for the preservation of genome integrity. The information from electron microscope showed that the telomere end in a loop structure; with a much larger loop called telomere (T) loop, and a much smaller loop called displacement (D) loops (Figure 1.2) [27].

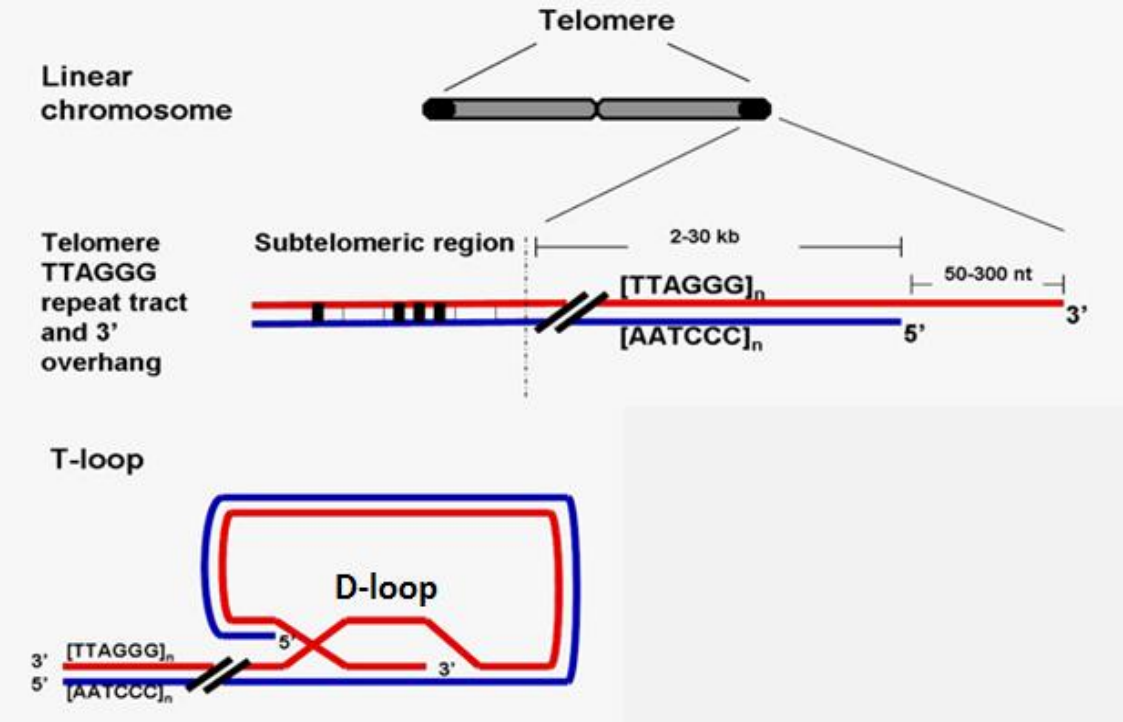

Figure 1.2 Telomeric DNA organization

Human telomeres at the chromosomal termini are the tandem repeats of TTAGGG for 2-30 kb with 50-300 bases hanging over their telomeric double strands. The structure at the end of telomere is a huge complex of a telomeric (T) loop and a displacement (D) loop. The folding back of telomere on itself constructs the T-loop while the displacement of 3' overhang into the double-stranded telomere forms the D-loop.

1.2.2 Telomeric protein

The lariat-like structure of the telomeric end is bound by telomere-specific proteins called shelterin or telosome and other non-specific telomere-associated proteins. Shelterin proteins specifically bind to both telomeric double strand region, the single-stranded G-rich overhang, and interact with other telomeric proteins. Thus, they play a crucial role in regulating the telomere length, the integrity, and the function of telomere [28].

In human, shelterin consists of six telomere-specific proteins: TRF1, TRF2, POT1, TIN2, TPP1, and Rap1. Three shelterin subunits, TRF1, TRF2, and POT1, directly recognize TTAGGG repeats. They are interconnected by the other three shelterin proteins, TIN2, TPP1, and Rap1, forming a complex that allows cells to distinguish telomeres from sites of DNA damage. Without the protective activity of shelterin, telomeres are no longer hidden from the DNA damage surveillance and chromosome ends are inappropriately processed by DNA repair pathways [13].

1.2.3 Functions of telomeres

The role in protecting chromosomes against recombination and fusion is the major function of telomeres. With the capping structure, telomeres prevent chromosome ends from being recognized by DNA repair systems. Telomeres also involves in homologous chromosome re-organization and meiotic recombination [29, 30].

1.2.4 Telomere shortening and replicative senescence

In 1961, the Hayflick limit was introduced to describe a cellular phenomenon called cellular senescence, a state of irreversible growth arrest [31]. Hayflick revealed that cultured human diploid cells could not proliferate continuously but stopped after dividing for a number of times. The cells were blocked at G1 while their metabolic functions are maintained. The specific characteristics of senescent phenotype are both morphological and biochemical changes, including the expression levels of p53, p16 and p21, and higher level of β-galactosidase, the lysozyme active at acidic pH. This cellular senescence is also considered as a tumor suppressor mechanism [32].

In the 1970s, James D. Watson defined the end-replication problem, which leads to telomere shortening in each cell division (Figure 1.3). Telomere shortening was postulated as a biological clock of cellular aging, which regulates the number of cell

divisions before encountering the state of growth arrest or replicative senescence. In somatic cells, the loss of telomere ranges from 25 to 200 bp per division [33, 34]. Telomere length has been purposed as an indicator of replicative history and the loss of telomere fosters cellular senescence. Nevertheless, the association between telomere attrition and entering senescence state is somehow discordant. Certain cells with shorter telomeres and some senescent cells with fairly long telomeres (5-10 kb) continuously proliferate. The discrepancy might be resolved by the fact that the shortest telomere, not the average short that triggers cellular senescence [25]. In aging cells, short telomeres initiate DNA damage signals through the activation of p53 to increase the background level of activated p53; thus, the threshold of senescence or apoptosis activation is lower. With this condition, the cells and tissues in the elderly are more sensitive to stress [35]. In germ line or in tumor cells, telomere elongation by telomerase provides a correction of telomere loss owing to DNA degradation or incomplete replication [36].

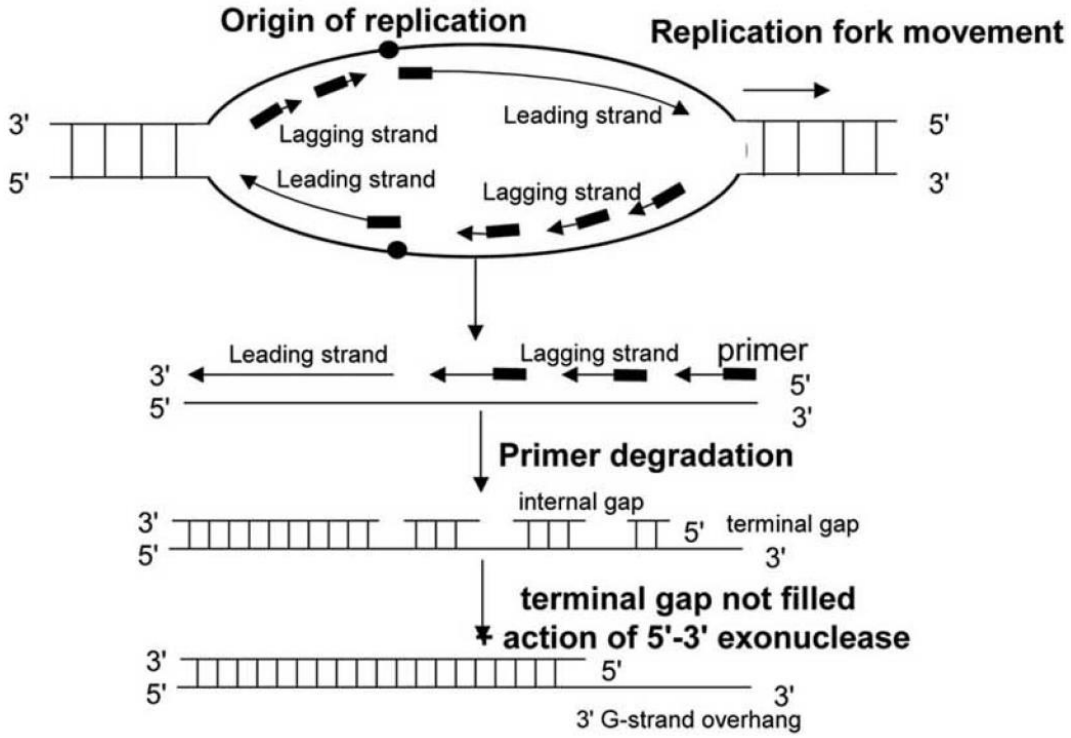

Figure 1.3 Telomere shortening during DNA replication

During DNA replication, the leading strand and lagging strand are newly synthesized only in the direction of 5’ to 3’ by DNA-dependent polymerases, which is unable to synthesize a DNA chain *de novo* without primers. When the synthesis is complete, the primers are degraded and the gaps between Okazaki fragments of the lagging strand and the space at the end of both strands are generated. Only the gaps, not the space are filled by the DNA polymerase. In addition, the space is further enlarged for 130-210 nucleotides by a putative 5'-3' exonuclease causing shortened 5’telomeric end and 3’ end hanging over.

1.2.5 Telomere maintenance

In cultured primary cells, telomere shortening regulates cell division for a certain number of passages as demonstrated by Hayflick in 1961. [32, 37]. However, in order to produce offsprings, reproductive cells must have a mechanism to maintain their telomeres. This telomere maintenance is achieved by telomerase, a specialized retrotranscriptase that consists of RNA template and catalytic protein subunits, among other telomerase associated proteins. Telomerase can elongate telomere on the linear structure of G-strand end. During embryonic development, telomerase is greatly expressed; however, the expression is repressed in most somatic cells few weeks after birth. The level of telomerase is maintained in highly proliferative cells such as stem and progenitor cells, lymphocytes, basal keratinocytes and cancer cells [38]. Besides telomerase, there is a telomerase-independent recombination mechanism to elongate telomeres called alternative lengthening of telomere (ALT), which exists in certain types of cancers [39].

1.2.6 Telomere length as biological markers

Since cellular replication, together with damaging factors such as oxidative stress, can promote telomere shortening; telomere length is believed to be a biomarker for biological aging. Several evidences show that telomere length from leukocytes can be used as a biomarker for cardiovascular diseases [40]. In patients with coronary artery disease and chronic heart failure, shorter telomeres are found to correlate with classical cardiovascular risk factors [41]. This association might be explained by the telomere shortening rate accelerated by cardiovascular risk factors [42]. A study in blood samples of the elderly revealed that those with shorter telomeres had almost twice higher risk in dying of heart disease or infectious disease [43]. In a psychological problem, a meta-analytic study revealed that patients with depression had shorter telomere lengths than healthy controls [44]. Telomere shortening is also found to be associated with cancers. A cohort study following 787 Italian people initially free of cancer showed that there were 3 times higher risk of developing cancer in subjects with the shortest telomeres than those with the longest telomeres [45]. Another example, a meta-analysis study in Caucasian and Asian subjects, retrospective designs, hospital-based controls and smaller sample sizes, revealed the significant association between shorter telomeres and overall cancer risk [46]. Moreover, a study about ovarian cancer revealed that shorter telomere

length, especially in the women under 30 of both familial and sporadic cases, was associated with increased ovarian cancer risk [47].

Different lifestyles also impact on telomere length. In people with leisure-time screen-based sedentary behavior, leukocyte telomere length (LTL) shortening was observed [48]. On the contrary, moderate physical activity levels could provide a protective effect on telomeres of peripheral blood mononuclear cells (PBMCs) [49]. Stress, even only a short period of 1 year, could accelerate the rate of LTL attrition, while the intervention with healthy behaviors could lessen the shortening rate [50]. In a pilot study, yogic meditation could improve mental health and increase telomerase activity of the family dementia caregivers with depressive symptoms [51]. The intake of sufficient impact nutrients also influences telomere length. For example, women who used multivitamin were found to possess longer LTL [52]. The interest in 'super old/ successful agers living longer than the average human life span (90 years of age or over) have divulged a great maintenance of genetic stability reflected by longer and/or more stable telomeres, which even ameliorated by a healthy lifestyle and in absence of diseases [53].

A study in mice revealed that the shortest telomere, not average telomere length, was critical for cell viability and chromosome stability [19]. They studied in 2 groups of mice both having short telomeres but with or without telomerase (mTR+/- and mTR-/-, respectively). Using quantitative fluorescence *in situ* hybridization (Q-FISH), mTR+/- mice had no chromosome ends without telomere repeats, while a significant number of short telomeres in mTR-/- mice was observed. Interestingly, the average telomere length in mTR+/- mice was only small different from that of mTR-/- mice. This result suggested that only shortest, not global telomeres were restored by telomerase. This study also found that shortest telomeres, especially on chromosome P arms, engage more possibility of chromosome fusion and the fusion junctions usually contained no telomere repeats. It implied that shortest telomeres might play a critical role in telomere dysfunction, which usually leads to cell cycle arrest and apoptosis. Another study on breast cancer showed that Chr17p telomere lengths in patients with breast cancer duct carcinoma in situ (DCIS) or invasive duct carcinoma (IDC) were shorter than in normal breast epithelial cells [20]. Compared to DCIS alone, the more progressive DCIS with IDC had more Chr17p telomere length heterogeneity. Interestingly, the rate of

shortening on Chr17p telomeres was greater than the average shortening of all telomeres. This study suggested that there might be mechanisms preferably dilapidating some telomeres faster while others were protected. In 1998, telomere lengths in cultured human fetal liver cells were studied by Q-FISH and terminal restriction fragment (TRF) analysis [21]. The study revealed that the flow cytometry-sorted Chr17 p telomeres were shorter than both the lengths of other sorted chromosomes and the average telomere length. This finding suggested that Chr17p short telomeres might impart in the frequent loss of Chr17p alleles in human cancers [54, 55]. With these reasons, telomere length has become one of the biomarkers for aging and the shortest telomere length might be a better longevity maker than the average one.

1.2.7 Principles, advantages, and limitations of techniques in assessment of telomere length

Several techniques have been applied to determine telomere length: for example, terminal restriction fragment (TRF) analysis, quantitative fluorescent *in situ* hybridization (Q-FISH), flow cytometry-based fluorescent *in situ* hybridization (Flow-FISH), real-time PCR, and single telomere length analysis (STELA) [56, 57]. TRF, Flow-FISH, or real-time PCR measures the average telomere length from all chromosomes, while Q-FISH or STELA provides the telomere length from an individual chromosome arm. The principle, advantages, and drawbacks of each method are concluded in Table 1.1.

Table 1.1 Principles, advantages, and drawbacks of the main telomere length measurement techniques [57].

Method	Principle	Advantages	Drawbacks
Terminal Restriction Fragment (TRF) analysis	In-gel hybridization or Southern blot of telomere repeats from digested DNA.	- Gold standard and widely used - Not require special reagents or equipment.	- Requires at least 1 µg for of DNA from many cells (~10^6) - Inaccurate length from subtelomeric polymorphism - Provides average telomere, not single telomere length - Labor-intensive and time-consuming
Quantitative Fluorescent *In Situ* Hybridization (Q-FISH)	Hybridization with fluorescent peptide nucleic acid (PNA) in metaphase cells and determine the intensity of signal by fluorescence microscope	- Provides both average and single telomere length - Provides the quantification of signal-free ends (<0.15 kb) - High accuracy.	- Requires both expensive equipment with external calibration and skilful operator - Requires a number of controls to prevent inter/intra-session variation - Labor-intensive and time consuming
Flow cytometry-based Fluorescent *In Situ* Hybridization (Flow-FISH)	Hybridization with fluorescent peptide nucleic acid (PNA) in interphase cells and determine the fluorescence signal by flow cytometer	- Simple - Quantitative and high throughput - High accuracy with reproducibility	- Limited to isolated and fresh cells. - Requires expensive equipment and skilful operator - Provides only average telomere length
Real-time PCR	Uses the ratio of telomere copy number to single copy gene copy number	- Simple and fast - High throughput	- Provides only average telomere length
Single Telomere Length Analysis (STELA)	Ligation PCR-based method.	- Requires no specialequipment. - Requires small amount of DNA	- Limited to well characterized chromosome ends: XpYp, 2p, 11q, 12q and 17p. - Have a bias on shorter telomeres and limited in detecting long telomeres (> 20 kb). - Labor intensive, time-consuming and low throughput.

1) Terminal restriction fragment (TRF) analysis

The primitive, well-known and widely used technique in measuring telomere length is TRF analysis; therefore, it is considered as the gold standard method. This method employs frequent cutting restriction enzymes to digest genomic DNA but leaves only telomeres from all chromosomes along with their subtelomeric regions, the region adjacent to telomere (TAS). The undamaged telomeres are resolved by agarose gel electrophoresis and detected by either Southern blotting or in-gel hybridization with a telomere-specific probe labeled with ^{32}P-radioactive or digoxigenin (DIG) molecule, depending whether the radioactive or non-radioactive system is used. The radioactive signal can subsequently be detected by autoradiographic film or phosphoimager, while the chemiluminescent signal generated from alkaline phosphatase linked anti-DIG antibody can be detected by X-ray film exposure. Due to the varying length of telomeres, the result is in smear pattern and its intensity and size is calculated according to a DNA marker to provide the mean TRF length. Due to the subtelomeric polymorphisms, this method can result in overestimating the length of telomeres. Moreover, the requirement of large amount of DNA ($\geq$1 ug) and its labor intensiveness are also problems.

2) Quantitative Fluorescent *In Situ* Hybridization (Q-FISH)

Quantitative Fluorescent *in situ* Hybridization technique is adapted to directly detect telomeres from cells in fresh (for chromosome-specific analysis), frozen, formalin fixed, paraffin embedded, or even permeabilized preparation by using a synthetic fluorescence-labeled $(CCCTAA)_3$ peptide nucleic acid (PNA) probe. The probe posses no charged phosphate backbones; therefore, its annealing efficacy is higher than DNA probes at low ionic strengths. Metaphase chromosome Q-FISH combines karyotyping technique to detect telomeres from each individual chromosome (p or q arm) of metaphase cells. It needs less cells (< 30 cells) than that of TRF assay (>10^5 cells). However, it is fairly labor-intensive and inappropriate for high throughput analysis. The small number of cells is required but difficult to get adequate metaphase cells at once. Besides, poor morphology of chromosome may occur from overexposure to high temperatures during sample preparation. A less labor intensive interphase Q-FISH is introduced to solve some drawbacks of Q-FISH by detecting telomere from interphase cells. However, only average length of telomere will be obtained, and 92 telomeres may

obscure one another and decline the efficacy in detection of individual telomere. Immunostaining technique can be applied to provide histological information of interested cells and this combination generates a new method called 'telomapping'. High-throughput (HT) Q-FISH is the next generation of interphase Q-FISH exploiting automatic fluorescent microscope to suit larger scale experiments.

3) Flow cytometry-based Fluorescent *In Situ* Hybridization (Flow-FISH)

Flow-FISH is another cytogenetic technique modified from Q-FISH in order to get a high throughput analysis of telomere length. Instead of using fluorescence microscope to detect fluorescent intensity, flow cytometer is used to quantify median fluorescence in a population of cells. This method eradicates the time required by Q-FISH to prepare metaphase cell spreads, and that flow cytometric analysis is also significantly faster than that of Q-FISH. Nevertheless, the drawback of Flow-FISH is that it cannot examine the telomere length of each individual chromosome as Q-FISH does. Besides, only fresh cells can be used for this method.

4) Quantitative (Real-time) PCR

The real-time PCR assay is another high-throughput method developed to measure telomere length from relative telomere-to-single copy gene (T/S) ratio, which is shown to be proportional to the mean TRF length in a cell. T/S ratio can be obtained from the proportion of telomere (T) and a single copy gene (*36B4*, *Albumin* or *Beta-globin* gene, S) signals. Several generations of this method have been introduced. In original one, the telomere and single copy gene are assayed separately; hence, the variation of the input amount of DNA sample affects the accuracy of the telomere length [58]. In 2009, the next generation was introduced to amend this variation by using multiplex PCR system. Thus, the quantities of both telomere and single copy gene are from the same sample DNA template [59]. Despite its high throughput, it needs standard graph of mean TRF length of a population to provide mean TRF length.

5) Single Telomere Length Analysis (STELA)

Single telomere length analysis is a DNA ligation and PCR-based method in assessment of telomere length from individual chromosome by targeting specific telomere end, which is impossible with TRF analysis. It employs the ligation of telolette linker to either 5' or 3' overhang telomeres before amplifying the product by PCR. The original C-strand STELA targets 5' telomeric end by using T4 DNA ligase to join 3' end of a telorette linker to 5' telomeric end. This can happen due to the 7 telomeric G-strand complementary bases of a telorette linker, which once anneals just next to 5' telomeric end will allow the DNA ligase to function (Figure 1.4) [60]. G strand STELA used in studying the 3' telomeric ending sequence targets 3' overhang by using a platform guide template, which once anneals at the very end of 3' overhang will allow G-telorette linker to anneal. The 5' end of annealing G-telorette linker will then be ligated to the 3' overhang (Figure 1.5) [70]. After the ligation, the product from both methods must be purified by phenol/chloroform extraction or DNA purification kit, and then amplified by using teltail primer (an oligonucleotide targeting telorette linker) and a primer specific to an interested chromosome arm. The PCR product is then analyzed by Southern blotting with ^{32}P-labeled telomere-adjacent probe hybridization. The radioactive signal is detected by phosphoimager or autoradiographic film. Although this technique is advantageous in access the length from individual chromosomes, it's a time-consuming method combining ligation and DNA purification steps. Moreover, being PCR-based, telomeres larger than 20kb cannot be amplified and there is a bias towards shorter telomeres.

1.3 Objective

To develop a less labor-intensive and non-radioactive method in measuring chromosome 17p telomere length.

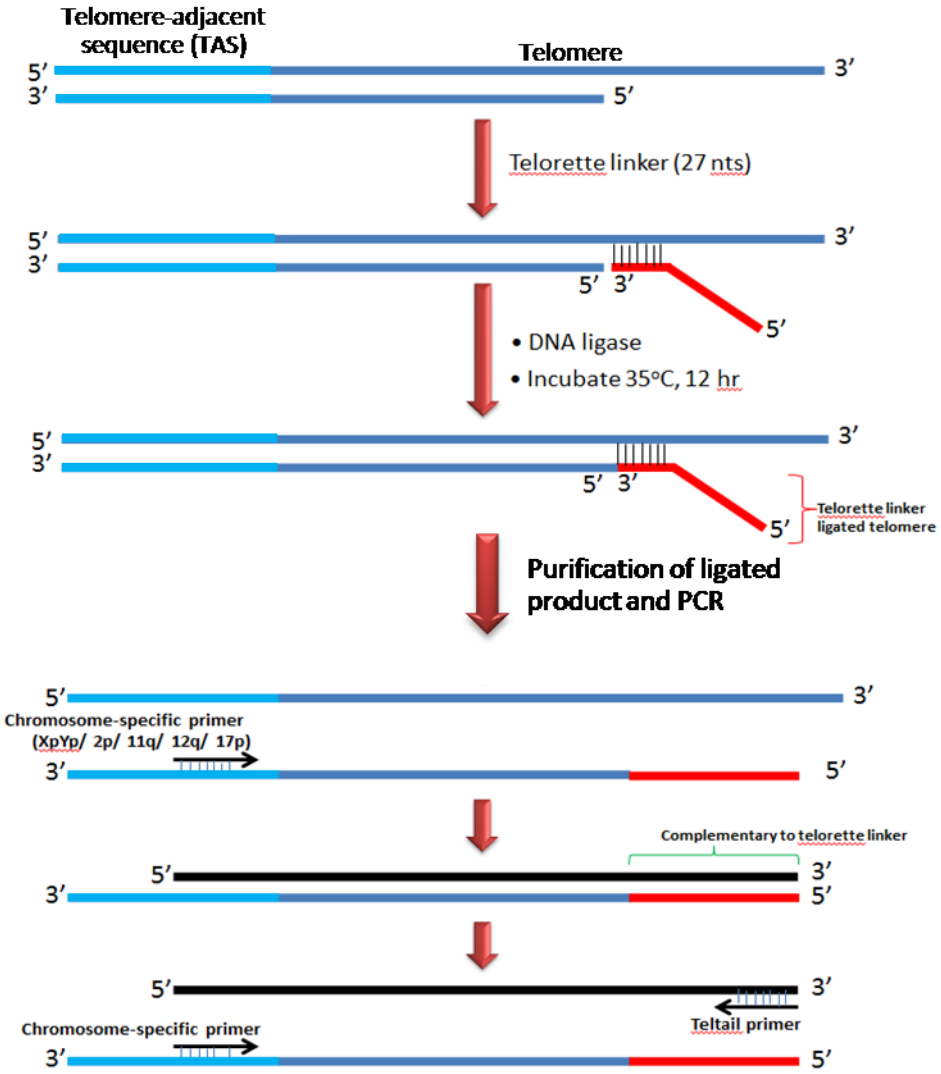

Figure 1.4 The principle of C-strand STELA

During incubation at 35°C for 12 hr in DNA ligation step, once 7 bases at 3’ end of a telorette linker complementarily binds at 3’ overhang of telomere just next to 5’ telomeric end, T4 DNA ligase will join the 3’ end of the telorette linker to the 5’ telomeric end to yield 5’ telorette-linked telomeres. In PCR step, the telorette-linked telomere of an interested chromosome is specifically amplified by a given chromosome-specific primer to produce chromosome-specific extended products (black line). Next, the extended products will be the template for teltail primer. With both chromosome-specific and teltail primers, chromosome-specific telomere will be multiplied.

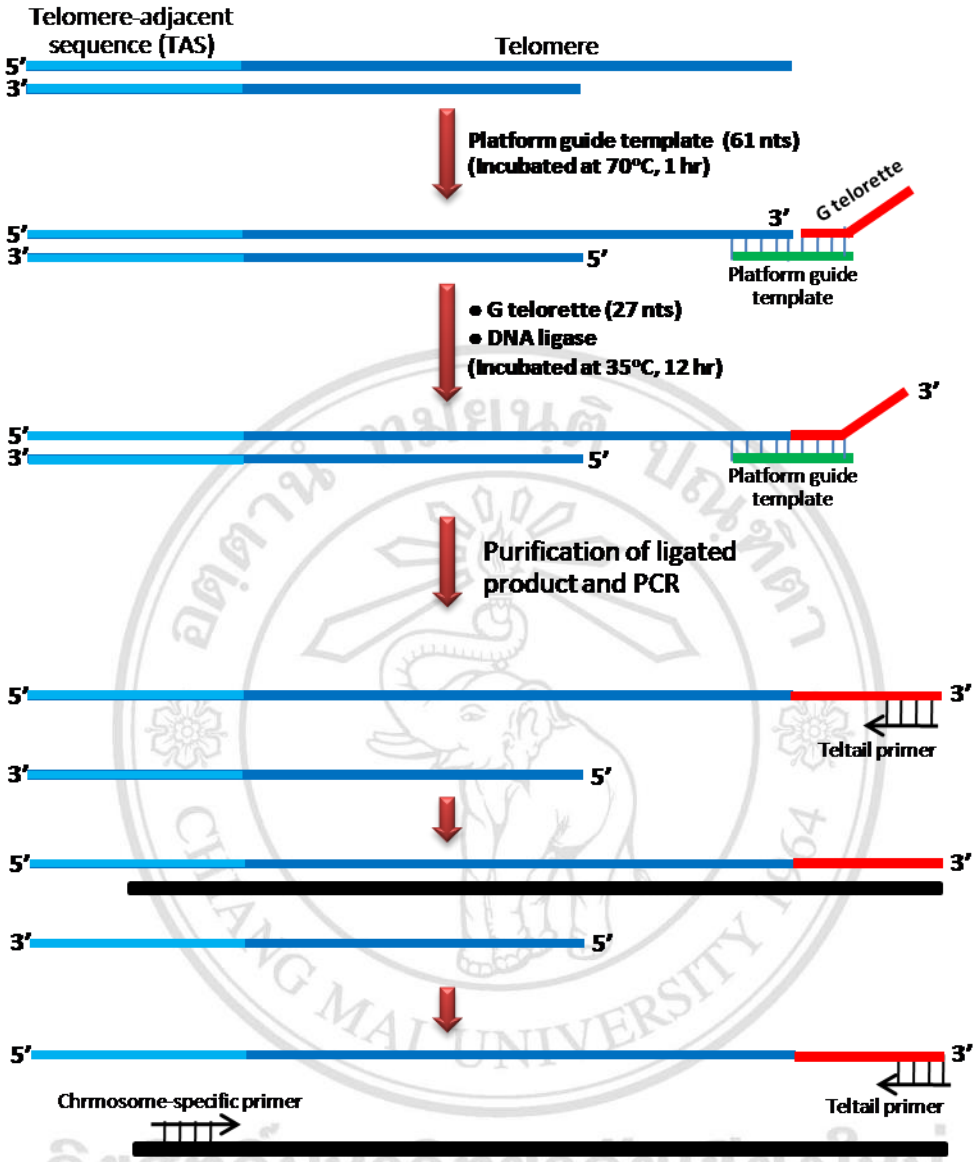

Figure 1.5 The principle of G Strand STELA

DNA is incubate at 70°C for 1 hr with the platform guide template, which once complementary binds at the end of 3'overhang will serve as a platform for a G telorette to anneal. The DNA ligase will then join the 5' end of the G telorette to the 3' telomeric end. After ligated DNA purification, the PCR will be used to amplify a chrosome-specific telomere by using a chromosome-specific and teltail primers.