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Chapter

Telomerase Structure and Function, Activity and Its Regulation with Emerging Methods of Measurement in Eukaryotes

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Abstract

The telomerase reverse transcriptase has an essential role in telomere maintenance which is very important in aging process and cancer biology. Recent studies have revealed three-dimensional architecture of both human and ciliate telomerase at about 25 Å resolution, using single particle electron microscopy (EM). Telomerase supplements the tandem array of simple-sequence repeats at chromosome ends to compensate for the DNA erosion inherent in genome replication which makes it to be distinct among polymerases. Telomeres are found at the end of eukaryotic linear chromosomes and proteins that bind to them and help to protect DNA from being recognized as double-strand breaks thus preventing end-to-end fusions. The activity of telomerase is tightly regulated at multiple levels of cellular development, from transcriptional regulation of the telomerase components to holoenzyme biogenesis and recruitment to the telomere site for activation and processing. Commonly used methods in telomere biology are telomere restriction fragment (TRF), telomere repeat amplification protocol (TRAP) and telomere dysfunction induced foci (TIF) analysis. This chapter summarizes our current knowledge on the mechanisms of telomerase recruitment and activation using insights from studies in mammals and budding and fission yeasts. Finally, we discuss the differences in telomere homeostasis between different cell types and non-telomerase telomere maintenance mechanisms.

Keywords: telomerase, telomere, holoenzyme biogenesis, chromosome ends, reverse transcriptase

1. Introduction

Telomerase is a ribonucleoprotein complex, composed of a reverse transcriptase enzyme catalytic subunit and a long non-coding RNA that contains the template sequence for telomere synthesis [1, 2] and is required for linear chromosome maintenance in most eukaryotes. The enzyme telomerase is active in germ cells and during early embryogenesis, ensuring restoration of telomere length for the next generation. However, when using an aged somatic cell with shortened telomeres for

cloning, the offspring might start with a diminished replicating capability of its cells and consequently age, or at least reach senescence, faster [3]. Telomere biology differ significantly among mammalian species, ranging from humans with very short telomeres and limited telomerase activity in the cells to mice with extremely long telomeres and active telomerase in multiple tissues. Human fibroblast cells have been reported to possess short telomeres and suppress telomerase activity [4]. Hence, human cells undergo permanent growth arrest, or replicative senescence, which is triggered by the critically short telomeres upon serial passaging in culture [5, 6]. Replicative senescence is an important barrier in tumor progression, as malignant tumors must reactivate telomerase or use the alternative lengthening of telomere mechanism to gain unlimited proliferation potential [7]. The expression of some components of the telomerase holoenzyme is tightly regulated [8]. For instance, in unicellular eukaryotes TERT and TER are constitutively expressed. In mammals, TERT is expressed only in highly proliferative cells and tumor cells. Somatic cells and cells with low proliferative capacity lack enzyme activity, this is the reason why telomerase activity is extensively studied as a potential target for antitumor therapy [9, 10]. However, apart from telomerase there are other mechanism used to maintain chromosome length; in some organisms, such as the fruit fly *Drosophila melanogaster*, retrotransposon-like elements are alternatively used to replenish the DNA at the ends of chromosomes [11]. As reported by Miriam Aparecida Giardini et al. [12], under certain circumstances, yeast and human cells that lack telomerase activity, as well as some telomerase-negative tumor lineages, are able to maintain their telomeres using a recombination-based DNA replication mechanism known as alternative lengthening of telomeres (ALT) [13, 14].

Research conducted in the past 10 years has revealed important discoveries on the evolution of telomere maintenance mechanisms. [15]. Telomeres serve as substrates for telomerase, the enzyme responsible for adding DNA to the ends of chromosomes, thus maintaining chromosome length [9, 16]. To compensate for the DNA erosion inherent in genetic stability, telomerase adds tandem array of simple-sequence repeats at the chromosome ends. The template for telomerase reverse transcriptase is within the RNA subunit of the ribonucleoprotein complex, this contains additional telomerase holoenzyme protein components within cells that assemble the active ribonucleoprotein and promote its function at telomeres. In terms of its reiterative reuse of an internal template, telomerase is different among other polymerases [17]. Like many polymerases, telomerase catalyzes nucleotide addition to a primer 3′ hydroxyl group, forming a product-template duplex. Accordingly, telomerase and other polymerases share a metal-dependent chemistry of nucleotide addition. Beyond these parallels, telomerase possesses unique properties of nucleic acid handling. Accurate telomeric repeat synthesis depends on strict boundaries of template copying within TER. Also, telomerases from most species studied have the exceptional ability to extend a primer by processive addition of repeats (reviewed in [17]). Repeat addition processivity (RAP) obliges dissociation of the product-template duplex without product dissociation from the enzyme [17]. The template-dissociated single-stranded DNA must maintain template-independent interactions while the template repositions for base pairing of its 3′ end, rather than the 5′ end, with the product. These coordinated nucleic acid handling events transpire as part of the full catalytic cycle of repeat synthesis [17].

Telomeres are specialized nucleoprotein structures located at the ends of linear chromosomes; they consist of TTAGGG repetitive sequences. They function to prevent natural chromosomal termini from activating the DNA damage response. [18]. The knowledge and understanding of telomerase structure, mechanism of action and factors involved in its activity would give more insight in overcoming the problem of replicative senescence.

2. Telomerase organizational architecture

During the last 4 years, progress in telomere research has revealed the three-dimensional architecture of telomerase in human and ciliate which is measured at about 25 Å resolution, this was obtained using single particle electron microscopy (EM). The structural analysis of the two holoenzyme complexes isolated from cells revealed that telomerase in ciliate is monomeric while the human telomerase is dimeric and it is only functional as a dimer [19]. Telomerase is a RNP complex with high-molecular weight and comprises of two major components and these are; TERC and a TERT (**Figure 1**) [12]. TERC is the RNA component which is essential for telomere synthesis; this serves as a template to elongate the 3′ overhang of the telomeric G-rich strand and specifies the repeat sequence added. In vertebrates, the TERC is comprised of three highly conserved structural domains and these are: the template pseudoknot domain, CR4-CR5 domain, and the small Cajal-body RNA domain. The template pseudoknot domain contains the template region for telomeric DNA synthesis and a conserved pseudoknot structure crucial for telomerase activity [20, 21]. Among eukaryotes the RNA component varies dramatically in sequence composition and in size [22–26].

TERT contains catalytic domains and is the protein component which acts as a specialized reverse transcriptase. In humans, TERT and TERC are the components required for telomerase activity in *in vitro* condition, although in *in vivo* condition some proteins are associated with the holoenzyme complex and are also essential for the catalytic function of telomerase enzyme [27]. About 32 different proteins are associated with human telomerase *in vivo* so as to maintain its functionality [28]; but few of these proteins are phylogenetically conserved. Proteins associated with telomerase activity have been best categorized in eukaryotes. Ciliate telomerase RNPs complex comprises a telomerase-specific La motif protein that folds telomerase RNA into a conformation that will be recognized by the TERT component [29].

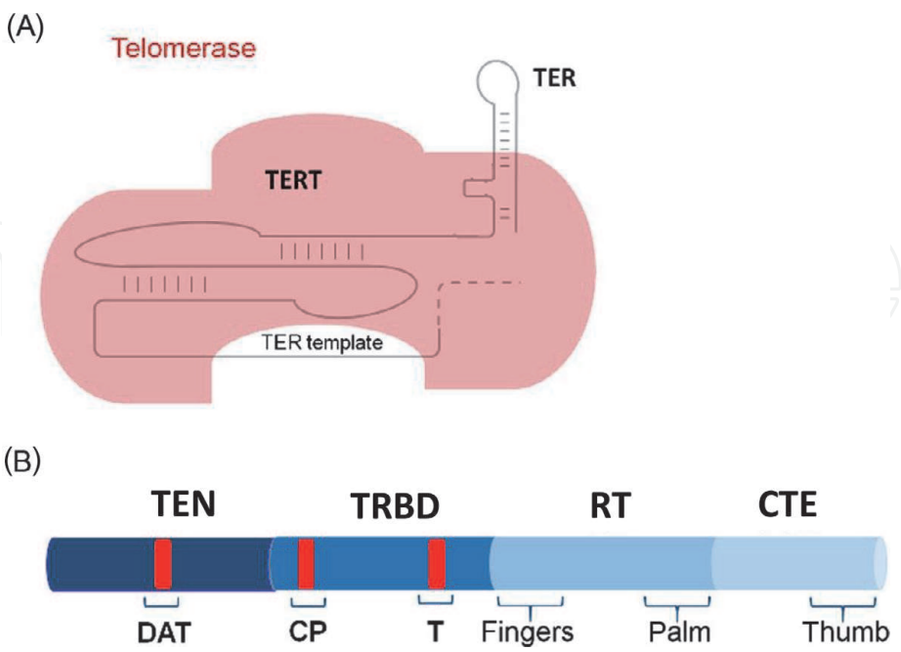

Figure 1.
Telomerase holoenzyme showing the various components. (A) Telomerase reverse transcriptase component (TERT) and telomerase RNA component (TER). (B) Diagram representing the TERT primary structure showing important TERT domains which include; the telomerase N-terminal domain (TEN), telomerase RNA-binding domain (TRBD), reverse transcriptase domain (RT) and C-terminal extension region (CTE). The position of the structural fingers, palm, and thumb subdomains are also highlighted. Openly accessed from Miriam Aparecida Giardini et al. [12] and Nanda Kumar and Cech [34].

At least two additional subunits, p45 and p75 are present in *Tetrahymena thermophila* holoenzyme which are not considered essential for TERT RNP assembly but are required for telomere elongation process. This common role is played by the proteins Est1p and Est3p in *Saccharomyces cerevisiae* [30]. There has not been any report in humans for proteins with this type of function, but known interaction partners of human telomerase RNA (hTR) have been reported and they include dyskerin (the H/ACA-motif RNA binding proteins), NHP2, NOP10, and GAR1. These four proteins assemble with hTR and with large families of H/ACA-motif small nucleolar (sno) RNAs and small Cajal body (sca) RNAs (**Figure 2**). Amino acid substitutions in dyskerin reduce hTR accumulation and this give rise to the X-linked form of dyskeratosis congenita (a bone marrow failure syndrome) [31]. Proteomics of highly purified active human telomerase led to the suggestion that only hTERT and dyskerin are associated with hTR [28]. However, this conclusion is challenged by previous studies showing that dyskerin possesses minimal RNA binding affinity in the absence of its H/ACA-motif binding partners NHP2 and NOP10 [32].

In general, telomerase RNP complexes exhibit conserved compositions and structures, even in evolutionarily distant organisms. Their compositions are similar from yeasts to mammals, including humans (**Figure 1**) [5, 12, 33].

For *in vitro* enzyme activity, minimal complex formation by TERT and TER components is sufficient. Nevertheless, *in vivo*, enzyme biogenesis, enzyme activity, and nucleotide addition processes also depend on other accessory proteins, indicating that a relatively complex maturation pathway is required for generation of an active RNP that has to find its substrate [35, 36]. Telomerase function to avoid the loss of terminal DNA, which is caused due to inability of DNA polymerases to completely replicate the 5′ ends of linear DNA molecules and also the actions of exonucleases involved. Both processes are responsible for generation of transient 3'OH overhangs found on the opposite ends of the leading and lagging DNA strands. The recognition of these overhangs are done by the end-binding proteins, they bind to the overhangs and afterward recruit telomerase to elongate the G-strand termini. The C-strand is synthesized by the conventional DNA replication pathway as soon as the telomeres are replicated by the telomerase [2, 14, 37, 38].

2.1 Telomerase-associated proteins

Though expression of hTERT and hTERC in rabbit reticulocyte lysates is sufficient to reconstitute basic telomerase enzyme [29], but the *in vivo* requirements for other factors necessary in the assembly of the active enzyme which did not clearly revealed some of this *in vitro* reconstitution, even though some of these factors are present in the rabbit reticulocyte lysates [39]. The molecular chaperones; Hsp90 and p23 are present in rabbit reticulocyte lysates. These are directly associated with the hTERT and are necessary for telomerase activity [39]. Biochemical and genetic studies reveal that additional protein subunits of telomerase exists which may be involved in the biogenesis or assembly of active telomerase RNP complex and may facilitate or regulate the access of telomerase to its substrate (i.e. the telomeres) [39].

2.2 hTERT-associated proteins

Biochemical fractionation of telomerase activity from the yeast *Tetrahymena thermophila* was used in identifying the first telomerase-associated proteins [29, 39]. The proteins, p80 and p95, were identified by their association with the RNA

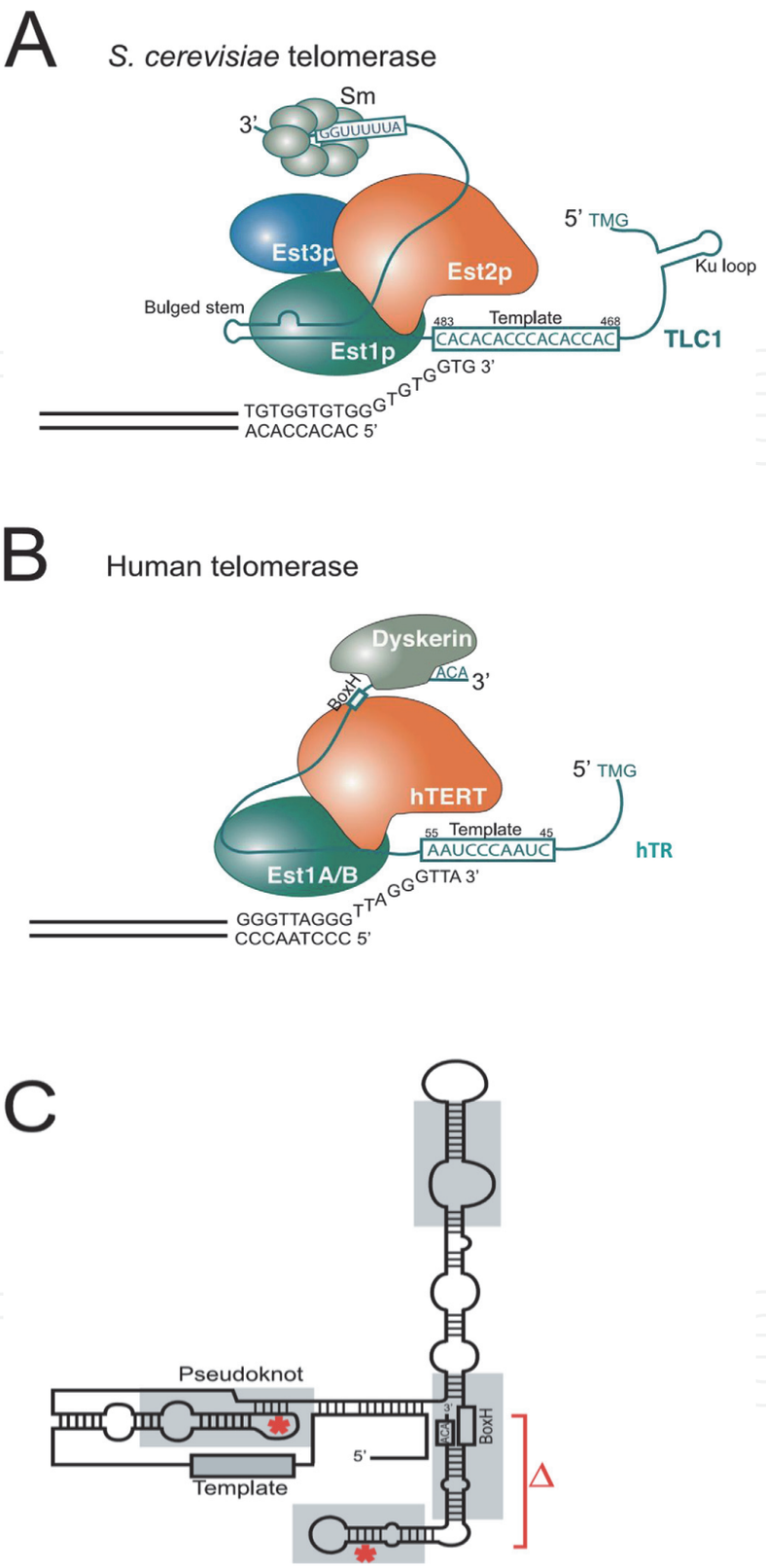

Figure 2.
*Showing schematic structure of (A) budding yeast (*S. cerevisiae*) telomerase, (B) human telomerase cropped at a telomere 3'end, and (C) vertebrate telomerase RNAs showing the conserved structural motifs. The positions of* DKC *mutations in the human telomerase (*hTERC*) gene are shown in red. Images are adopted from Smogorzewska and de Lange [33].*

component of telomerase and by copurification of telomerase activity [37]. In a similar report, *Tetrahymena* strains were shown to lack p80 and p95, and the levels of telomerase activity with its RNA appear to function totally normal. This suggests that these proteins are not core components of telomerase and can be a separate

ribonucleoprotein that was copurified nonspecifically with telomerase [40]. Nevertheless, it was reported in another study that cells devoid of p80 and p95 have their telomeres elongated both in macronuclei and micronuclei but lose genetic content in their micronuclei, which suggest the role of p80 and p95 proteins in micronuclear genomic stability and telomere length maintenance [40].TEP1 (telomerase-associated protein 1) which was identified in humans, mice and rats is the mammalian homolog of p80, and is involved with telomerase activity. [39]. TEP1 consists of 2629 amino acids, much larger than p80. About 900 amino acids found at the amino terminus of TEP1, contain region homologous to p80 which were found to associate with telomerase RNA. The carboxyl terminus of TEP1 contains 12 WD40 repeats, a motif known to be involved in protein-protein interactions [39]. TEP1 expression can be distinguished in most tissues irrespective of telomerase activity. Disturbance of mouse TEP1 has no effect on telomerase activity or telomere length in spite of its association with both the RNA and catalytic components of telomerase in cell extracts from immortalized human, mouse, and rat cells [39, 41]. The TEP1 protein has also been recognized as a constituent of large cytoplasmic particles called vaults, which are ribonucleoprotein complexes [39]. The functions of TEP1 in both telomerase and vaults are still not elucidated [39]. The molecular chaperone p23 was first identified to be associated with hTERT using the amino terminus (amino acids 1–195) as the desirability in a yeast two-hybrid screen. Consequently, it was observed that the proteins p23 and p90 were in association with hTERT in mammalian cells and in *in vitro* condition [41]. The first identified sets of proteins which interact physically and functionally with human telomerase is the hsp90 chaperone complex and have been found to support complete assembly of ribonucleoprotein and the formation of active telomerase enzyme [39]. It is well known that other reverse transcriptase that are of viral origin also interact with hsp70, hsp90, and p23, but appear to be transient [12].

3. Telomerase activity in different cell types

Telomeres are needed to maintain the ends of chromosomes and sustain chromosome stability in eukaryotic cells. Telomeres loss their noncoding DNA sequences in the erosion that happens during DNA replication in each cell cycle. They do this to protect the genetic information in the chromosomes [42, 43]. Most somatic cells enter into replicative senescence because they have undergone sufficient cell divisions to cause critical shortening of the telomeres. Some cells, including lymphocytes, germ cells, stem cells and unicellular eukaryotes such as yeast, express the enzyme telomerase, which gives them the ability to replenish their telomeres and give them further replicative potential [30, 44]. Most human tumors express active telomerase enzyme making them immortal while in differentiated cells, expression of the telomerase components is closely regulated [45, 46]. A direct correlation between continuous cell division and telomere length maintenance was studied in *in vitro* culture condition through ectopic expression of telomerase activity in somatic cell [47]. Even though cancer cells steadily maintain telomere length which also tend to be shortened in later stage [48, 49], some of them are critically shortened and are termed ‘t-stumps’ [50] resulting in immortal cells which possessed a high risk of chromosome instability. This is extraordinarily different from our understanding of telomerase activity in normal cells, in which telomerase acts to elongate shorter telomeres until they are no longer short [7, 51]. The reason why telomerase behaves differently in cancer cells still remains an area of interest for research.

3.1 Telomerase expression and cellular proliferation

The expression of telomerase enzyme activity in different types of cells has been characterized using the telomeric repeat amplification protocol (TRAP) assay. The method fundamentally measures the telomerase activity confined within a cell lysate *in vitro* culture [10]. By using this assay, it is well documented that most differentiated somatic cells lack detectable telomerase activity [10, 46], explaining the reason why telomeres shorten in each cell divisions [47, 48, 52]. In adult testes and ovaries, telomerase enzyme is highly expressed thereby, allowing consistently longer telomeres to be inherited by the next generation [48, 53]. During the early embryonic development, telomerase enzyme remains active but its expression declines after the blastocyst stage and cannot be detected in neonatal somatic cells [53–55]. Telomerase activity is weak in most stem cell populations [10, 44, 46, 56], this is not sufficient to immortalize cells but can extend the proliferative capacity of these cells (reviewed in [6, 57]). Remarkably, the Hayflick limit of somatic cells can be indefinitely avoided when telomere length is maintained by high expression of telomerase activity [47, 58]. Therefore, the level of telomerase activity and its expression determines the level of telomere length elongation and proliferative ability of a cell.

4. Regulation of telomerase activity in mammals

Telomerase activity is widely regulated owing to its important role in the maintenance of genome integrity. Multicellular organisms display tissue-specific, developmental and stress response strategies for telomerase suppression [59, 60]. In human somatic cells the inactivation of telomerase enzyme and maintenance of telomere length have been proposed to play a role as a tumor suppressor mechanism [61, 62]. This may also be needed for cell latency, differentiation, and death of some cell types [63]. However, collective telomere erosion limits the self-renewal ability of highly proliferative human cell lineages in the skin and blood [29]. The expression of TERC is universal while TERT expression is highly regulated in some organisms, especially in mammals. Many strategies have been proposed to control telomerase activity, because the enzyme can be regulated at various levels including expression level. For instance, the epigenetic modification of histones can modulate chromatin structure and the accessibility of the transcriptional machinery to regulatory regions of target genes. In this regard, numerous transcription factors, such as c-MYC, SP1, MAD1, and HIF-2a, have been shown to recruit either histone acetyltransferases or histone deacetylases to the TERT promoter to control TERT expression [64, 65]. However, the transcription expression is not constantly linked with the enzyme activity, which might result in transcription modulation failure [66]. Consequently, telomerase is expressed in embryonic stem cells, but TERT expression and telomerase activity are frequently very low or undetectable in somatic cells [67]. In contrast, telomerase activity seems to be high in most (85–90%) cancer cells [10, 46, 68]. Nevertheless, some cells that lack telomerase activity still exhibit a high level of hTERT transcription. In these cases, regulation at the level of alternative splicing leads to the skipping of exons that encode reverse transcriptase function [69]. In mice, the deletion of either TERC or TERT can result in telomere shortening, genomic instability, aneuploidy, telomeric fusion, and aging-related phenotypes [41, 70]. Therefore, telomerase dysfunction may lead to defects in various highly proliferative cells/tissues, ultimately leading to aging-related degenerative diseases [71]. The overexpression of TERT can dramatically increase the life span of mice in the background of the overexpression of tumor suppressor genes, such as p53, p16, and p19, indicating that TERT must have an anti-aging activity in mammals [4, 15, 72].

4.1 Telomere replication in the absence of telomerase

There exist some alternative mechanisms which are activated to maintain telomere length in the absence of telomerase activity. These mechanisms are principally based on recombination events that come into play to amplify or reorganize previously existing telomeric sequences [73, 74], and the mechanisms seem to be complementary to both the telomerase method and the method occupied in "retro transposition" [11]. The alternative mechanisms were first observed in budding yeasts that were able to survive and achieve telomere elongation despite lack of a functional telomerase [12]. Thereafter it was verified that this phenomenon is dependent on RAD52 (a protein involved in homologous recombination) [74]. Telomere lengths are also maintained by telomerase in most cancer cells, [46]. Reports have shown that approximately 10–15% of cancer cells elongate their telomeres by using one or more alternative mechanisms referred to as alternative telomere lengthening (ALT) [68, 75]. In the same way, immortalized cells can also elongate their telomeres using either telomerase [76] or ALT [77].

Other telomere-lengthening mechanisms also exist in the absence of telomerase activity. These mechanisms have been reviewed in details in previous reports [11]. The mosquito fly *Anopheles gambiae*, the vinegar fly *Drosophila melanogaster*, and some species of plants are other examples of organisms that use alternative telomere elongation mechanism by using recombination [11]. For instance, *Drosophila*, lacks telomerase activity and exhibits long tandem arrays composed of three non-LTR retrotransposons, HeT-A, TART, and TAHRE, instead of simple telomeric repeats unlike in most organisms. These were the first transposable elements revealed to play an important role in cell structure [11, 40, 78]. In *Trypanosoma brucei* (a haemoparasite), critically short telomeres generated by knocking out the TERT gene were stabilized by an unknown mechanism [79]. These short telomeres lack active transcriptional factors and tend to shorten more and more without leading to cell senescence due to their stability regardless of the absence of active telomerase enzyme [80, 81]. The mechanism by which these short telomeres are stabilized has not yet been revealed, but it is known that the telomerase-deficient strains switch variant surface genes (VSG) by duplicative gene conversion, which occurs more frequently than in wild-type strains and exhibit longer telomeres. Furthermore, it was observed that shorter chromosomes at no time underwent fusion and that telomere stabilization was sufficient to preserve genomic integrity, with no apparent effects on long-term population growth [82].

4.2 Methods of measuring telomerase activity

Methods used for the detection of telomerase activity can be divided into two major groups as described by Skvortsov et al. [83]: those based on direct detection of telomerase products, (**Table 1**) and those based on different systems of amplification of the signals from DNA yield from telomerase (**Table 2**). The methods discussed in this chapter (**Figures 3** and **4**) are suitable for testing telomerase activity in different types of samples such as; in protozoa, mammalian cells, mixed cellular populations, and tissues [83].

4.2.1 Methods containing the amplification of telomerase-synthesized DNA with modifications to the original TRAP

Telomeric repeat amplification protocols (TRAPs) are the most common methods employed for detection of telomerase activity which permit one to carry out semi-quantitative and quantitative analyses, by introducing some modifications [83].

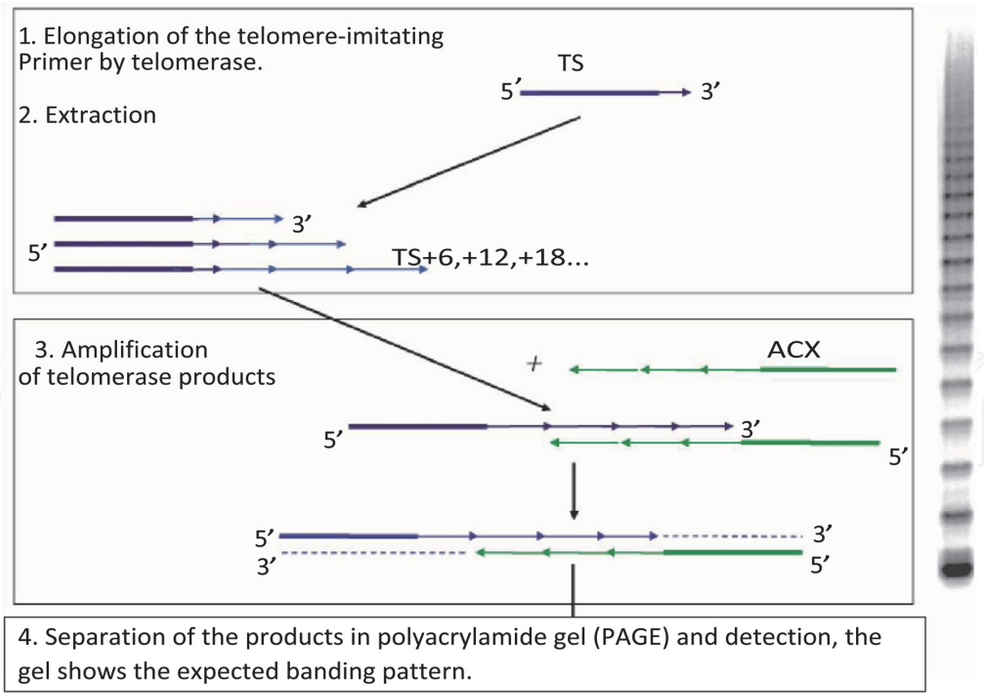

Figure 3.
Illustration showing the original telomeric repeat amplification protocol (TRAP) assay [83].

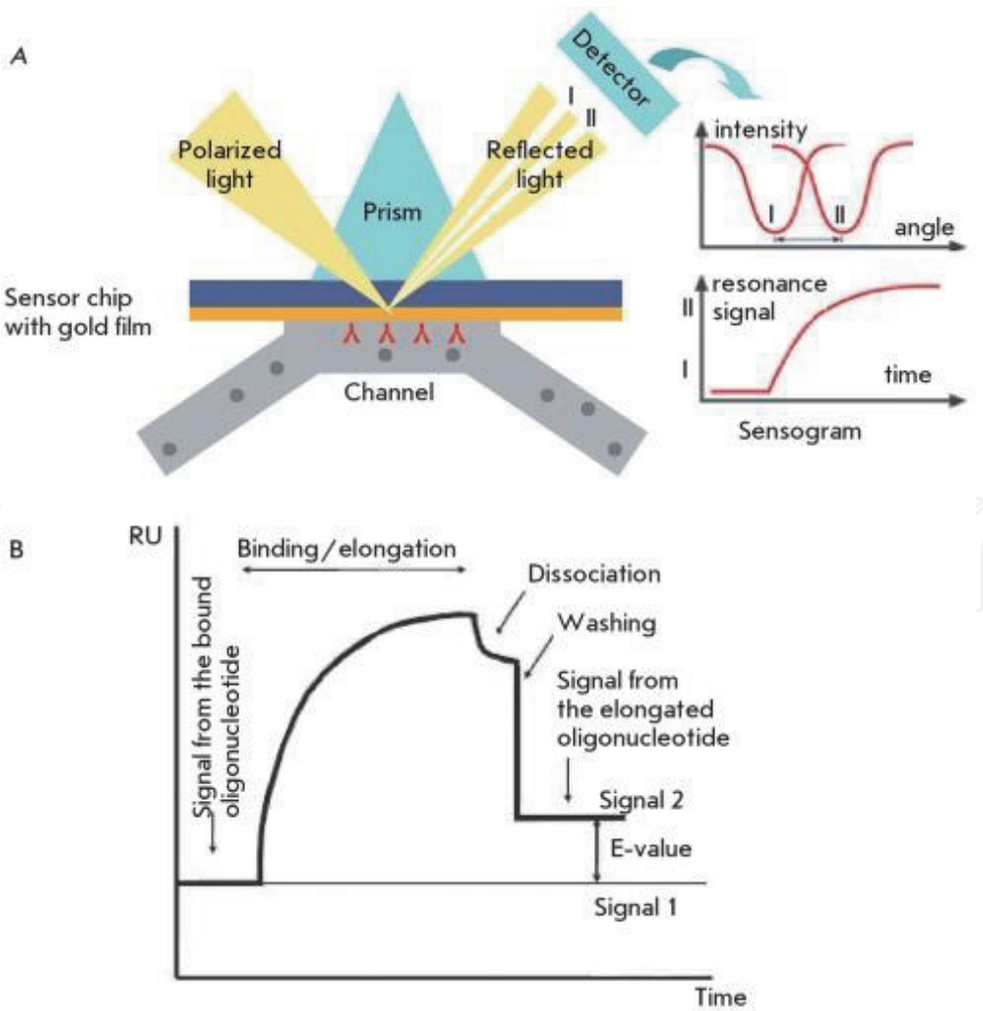

Figure 4.
Showing direct detection of telomerase by surface plasmon resonance (SPR) for detecting macromolecules; (A) sensogram corresponding to the general scheme and (B) SPR sensogram for telomerase activity detection. RU, resonance units. The difference between signals 1 and 2 represents DNA which was synthesized by telomerase [83].

Assay name	Description of modification of original assay	Benefits of modification	Potential limitation	References
Purification of telomerase-synthesized DNA and TRAP efficiency	This method involves the extraction of telomeric-synthesized DNA using modified magnetic beads where PCR inhibitors are eliminated or completely diluted. There are three main stages involved in the extraction process; the elongation of the substrate-imitating oligonucleotide by telomerase, the extraction of telomerase synthesized DNA using modified magnetic beads and finally the amplification step	Sensitivity towards PCR inhibitors is lower as compared with the standard TRAP method and its efficiency is slightly higher when analyzing tissue and other complex specimens	False-positive or false negative results can be obtained, and they may affect the validity of the diagnosis and prognosis of a disease. The nature of specimens, such as large volumes of fluid (blood, etc.) or presence of numerous normal cells, may also complicate the detection of telomerase activity	[83, 92]
Internal standards for TRAP	Here, the internal standards are amplified with the same primers as the telomerase-synthesized DNA. This permits the presence of Taq polymerase inhibitors (such as gem-containing compounds) in the samples to be analyzed and for performing of total control of PCR. The two most common standards used are of 36 and 150 bp in length	The 150 bp standard is more sensitive towards the Taq polymerase inhibitors present in the reaction mixture	There are two most common standards used, with the length of 36 and 150 bp. The 36 bp standard is excessively amplified if the specimens have a low telomerase activity, competes with the telomerase-synthesized DNA, and provides a false negative signal	[83, 90, 93]
"Two-primer" TRAP (TRAP) with an additional specific reverse primer	The telomerase-synthesized DNA is amplified by the use of two reverse primers with lengths of 20 (RP) and 38 (RPC3) nucleotides, in the presence of [3 H]TTP or [α-32P]dCTP	This modification of the standard TRAP is used to reduce false signals. Here there no electrophoretic analysis of PCR products but instead the total radioactivity is estimated as a decisive factor of telomerase activity		[83, 94, 95]
TRAP with fluorescence resonance energy transfer (FRET)	This method uses primers with an energy transfer (amplifluors) property instead of the normal primers used for amplification of the telomerase-synthesized DNA	The advantage of this modification is that the replacement of the telomerase substrate and reverse primer by amplifluors allows to achieve an increasingly high intensity of the fluorescent signal		[93, 96, 97]
TRAP with detection using a Scintillation proximity assay	This method uses scintillation proximity assay for the detection of amplified DNA instead of PAGE in TRAP. This modification helps to increase the detection rate of	In combination with the traditional TRAP, it helps increase the rate of detection of telomerase activity	The major disadvantage of this method is the use of tritium, and also like most methods for detecting TRAP products without PAGE, it is sensitive to PCR artifacts	[83, 98]

Assay name	Description of modification of original assay	Benefits of modification	Potential limitation	References
	telomerase activity. 5'-Biotin conjugated oligonucleotides are used as substrate in this method			
TRAP with detection using the hybridization protection assay (hybridization protection assay-TRAP)	In this method, after PCR, the amplified product is detected by the use of probes labeled with covalently bound acridine	This modification is safer and easier to detect telomerase product		[99]
TRAP with ELISA (enzyme-linked immunosorbent assay	In this method, after the amplification, concentration of DNA calorimetrically, enhancing the qualitative and semi-quantitative assessment of telomerase activity	TRAP-ELISA method is faster as compared with TRAP, which is based on the separation of the amplified DNA in gel. This makes it possible to use the TRAP-ELISA method in screening studies	One of the drawbacks of this method is the complexity in separating the telomerase-positive and telomerase-negative controls, which may result from the absence of internal controls and two steps of signal amplification	[100, 101]
TRAP with electrochemical detection	This method involves the treatment of the remaining PCR products after the initial PCR with 3 M HCl making it possible to determine dGMP (one of the products of the complete hydrolysis of the amplified DNA)	This modification has no special advantages over other methods in which no gel-electrophoresis or radioactive labeling are used	This method is more laborious and time consuming	[102]
TRAP with real-time PCR	This method employs the use of real time PCR which permits the simultaneous amplification of DNA and measurement of the amount of amplified products after each cycle. Combination of real time PCR with the standard TRAP makes it possible to obtain quantitative results and is suitable for analysis of large samples	This modification allows to obtain quantitative results. This modification is also suitable for studying telomerase inhibitors and analyzing large specimen series. The advantages of this method include; a high rate of detection of telomerase-synthesized DNA upon flow analysis and enhanced specificity due to the use of corresponding probes	Over-estimation of telomerase activity and leveling of small differences in the activity is possible without the assessment of the amount of real-time PCR products, due to saturation of the PCR reaction in the final stages. Also, there is the possibility of dimer formation resulting in false-positive signal	[83, 87, 103–105]
In situ TRAP	In this method, FITC-labeled primers (direct and reverse) are used. Here the fluorescence intensity and its localization in nucleus or cytoplasm are used to determine telomerase activity in separate cell types within a mixture. High telomerase activity in urogenital and bronchial lavages manifests	This modification allows for a semi-quantitative determination of telomerase activity and its localization in isolated cells of tissues and cell suspensions	This method is only appropriate for cancer cells and not suitable to study cell senescence	[106–111]

Assay name	Description of modification of original assay	Benefits of modification	Potential limitation	References
	itself through bright fluorescence of the nuclei of malignant cells. The signal from cytoplasm is detected in granulocytes and macrophages			
TRAP on microchips	This method involves the combination of two primer—TRAP and binding of PCR products on chips, followed by probe hybridization and detection using different fluorescent labels to determine telomerase synthesized DNA and the internal standard; for example, Cy3 for the telomerase-synthesized DNA being amplified and Cy5, for the standard	The modification is that; the introduction of microchips and hybridization using different probes for the detection of telomerase synthesized DNA		[83]
Transcription amplification of telomerase-synthesized DNA	In this modification PCR is replaced by transcription amplification, this is to increase the amount of telomerase-synthesized DNA. Combined with "hybridization protection," this method allows to determine the telomerase activity within short time period. The method's sensitivity allows to detect telomerase activity in specimens consisting of 1–1000 cells and also sensitive to the presence of RNases	The major advantage of this method over TRAP with PCR amplification is that no heating of a specimen is required upon amplification and that the specific Taq polymerase inhibitors are neglected. This method makes it possible to semi-quantitatively determine the telomerase activity in tissue and cell line extracts		[83, 87, 112]

Table 1.
Different modifications of TRAP method for telomerase activity detection.

Assay name	Description of modification or original assay	Benefits of modification	Potential limitation or disadvantage	References
Direct incorporation of a radioactively labeled substrate	This method is based on the ability of telomerase to elongate oligonucleotides in the presence of dNTP. The oligonucleotide or the dNTPs are radioactive labeled allowing the detection of telomerase activity electrophoretically by its incorporation into the telomerase-synthesized DNA	The modification allows for qualitative determination of the activity and processivity of telomerase in cell line extracts. Absence of artifacts associated with PCR. The telomerase-synthesized DNA can be immediately observed in the gel. Its size and amount can be estimated	The major disadvantage of this method includes the use of large amounts of radioactive isotopes with longer exposure time and insufficient sensitivity	[83, 113]
Determination of telomerase-synthesized DNA by changes in surface plasmon resonance	Surface plasmon resonance (SPR) is used to detect telomerase activity as the corresponding elongation of a telomere-imitating oligonucleotide, using a biosensor (5'-biotin-conjugated) oligonucleotides, which contain telomeric repeats, these are in situ immobilized on the surface of a dextrane sensor pre-treated with streptavidin on a BIACORE instrument	This method allows to quantitatively determine telomerase activity in tissue and cell line extracts and collect data on the kinetics of the reaction, demonstrating the binding and dissociation of telomerase from the substrate	The necessity of the BIACORE system and biotin-conjugated primers	[83, 114, 115]
Oligo-modified magnetic particles and NMR	This method involves the use of magnetic particles of an iron oxide which are modified with oligonucleotides that are complementary to telomeric repeats. These particles bind to the telomerase-synthesized repeats due to complementary interactions and form extended linear structures (MRS complexes). The local distortion of the magnetic field increases on nanoparticles in ordered ensembles, whereas the nonordered nanoparticles provide a considerably lower magnetic effect	This modification made it possible to analyze large number of specimens within short period of time (in several 10 of minutes) with high sensitivity. There also absence of artifacts which were associated with PCR. Radioactive label and PAGE are not required	In addition to the common instruments and reagents, these analyses require a plate NMR-spectrometer and a specimen of oligonucleotide-modified nanoparticles which are expensive	[83, 116]
Quartz crystal microbalance technique	An Au-quartz resonator can be used for a microgravitometrical analysis of telomerase activity, according to the quartz crystal microbalance technique. Here, in this method, the telomere-imitating oligonucleotide is bound to the surface of the sensor and is elongated by telomerase	The method allows for the quantitative determination of the telomerase activity in tissue and cell line extracts. The sensitivity threshold is up to 3300-cell extracts of the telomerase-positive cell line at a high rate	A frequency analyzer and an Au-quartz crystal need to be provided in this method. Also, the identification of artifact signals is complicated	[117]

Assay name	Description of modification or original assay	Benefits of modification	Potential limitation or disadvantage	References
Biobarcode assay for telomerase activity detection	In the original biobarcode system, magnetic particles bind to a target, which in turn binds to nanoparticles covalently modified with an oligonucleotide due to antigen–antibody interactions. DNA nanoparticles consist of gold nanoparticles and oligonucleotides of two types; one of these can form a duplex with the telomerase-synthesized DNA	This modification makes it most sensitive for direct detection of telomerase activity without amplification of the telomerase-synthesized DNA. Whereas the second cannot. Because of this, the probability of binding another DNA-target to the same nanoparticle is reduced. The electroactive complex $[Ru(NH_3)_6]^{3+}$, which is capable of binding to negatively charged DNA chains due to electrostatic interactions, is used for detection	A modified electrode and nanoparticles coated with oligonucleotides of two types are required	[118]
Optical biosensors assay	The principle of this method is similar to that of SPR. The method is based on the fact that upon binding of a target, the refraction index on the sensor's surface changes in proportion to the amount of bound targets	In this modification a cassette consisting of three oligonucleotides helps to avoid steric impediments. Phosphate groups covalently interact with the surface via the 5′-end of an oligonucleotide. Then, an oligonucleotide containing a short noncomplementary region on its 3′-end complementarily binds to the immobilized DNA. The prominent 3′-end of the DNA is modified with phosphorothioate, which enhances the affinity of telomerase-primer binding by a factor of 10	Phosphorothioate-modified oligonucleotides and a special optosensor are required. The low sensitivity for clinical samples	[83, 119]
Quantum dots telomerase detection	This method is based on a telomere-imitating oligonucleotide modified at its end with the thio group is attached to a nanoparticle (quantum dot). This quantum dot is capable of fluorescing by absorbing a quantum with a wavelength of λ_1 (400 nm) and emitting a quantum with a wavelength of λ_1' (560 nm)	This modification allows for a quantitative assessment of the telomerase activity	The disadvantage is that the sensitivity threshold is approximately 10,000 HeLa cells which is not enough for an analysis of clinical materials	[120]
An on-chip nanowire sensor assay	This method involves the use a sensor chip (a transistor comprising antibody-coated silicon nanowires with aldehydes groups on their surface, to which monoclonal antibodies can be linked) to determine telomerase activity, the transistor nanowire is modified by telomere-imitating oligonucleotides instead of antibodies.	This modification allows to determine telomerase activity in cell line extracts even though, it has not been tested on clinical materials. Its sensitivity is sixty five (65) T293 cells and the major advantage of this method is that a large number of different analyses of one specimen can be carried out on a single chip,	Here a transistor chip and the equipment to analyze it are required	[121, 122]

Assay name	Description of modification or original assay	Benefits of modification	Potential limitation or disadvantage	References
	After the introduction of telomerase and dNTP, the oligonucleotides bound on the surface elongate, which results in change in the conductivity of the transistor to which the oligonucleotides are bound	which can be a set of sensors with respect to various markers. Moreover, the stages of telomerase binding and dissociation can be observed		
Bioluminescence method	Bioluminescence method is used for the determination of telomerase activity based on the fact that the telomerase-catalyzed elongation of the telomere-imitating oligonucleotide is accompanied by the cleavage of pyrophosphate; its amount is determined luminometrically	The advantage of the method is the linear dependence of the signal on the amount of telomerase-synthesized DNA, combined with high efficiency. The sensitivity and specificity of this method can be comparable with the sensitivity of TRAP-ELISA	A luciferase system for bioluminescence detection and a luminometer are required	[123]
Electrochemiluminescence method	Telomerase activity can also be determined by electrochemiluminescence (luminescence upon electrolysis). In this method, the 5′-biotin-conjugated primer is elongated by telomerase followed by incubation with a suspension of magnetic beads modified with avidin	This method allows for the quantitative determination of telomerase activity in samples that contain at least 500 HeLa cells. It provides an appreciably high signal/noise ratio due to the stage of magnetic bead extraction; however, it has not been tested on clinical materials	Difficult to synthesize a sample, requirements to the equipment	[83, 124]
FRET and total internal reflection fluorescence microscopy method	This is a FRET-based method that is intended to distinguish the single-letter synthesis (a nonprocessive method of synthesis) and the beginning of synthesis of the second DNA repeat (a conditionally processive method of synthesis) by individual complexes of *Tetrahymena thermophila* telomerase. Biotin-conjugated primers (TG)8 T2 G4 T2 were used on streptavidin-coated quartz slides which were treated with telomerase-containing extract in the presence of dGTP and ddTTP	The method allows to identify individual signals from the elongation of the primer by telomerase and can be combined with FRET-based methods to investigate telomerase structure	This method can be used to determine only the first 1.5 telomerase-synthesized repeats; that is, it does not reveal total telomerase activity. The method has been tested only on *T. thermophila* telomerase and has been aimed only at solving research problems till date	[83, 125]

Table 2.
Methods for direct detection of telomerase-synthesized DNA.

Assay name	Description of method	Advantage of assay	Potential limitation/disadvantage	References
Telomere restriction fragment (TRF) analysis	Telomere restriction fragments (TRF) involves Southern blot hybridization using probes against telomere repeats used for the analysis	This technique is widely used and it requires no special reagents or equipment	Quantification is very difficult. It requires large number of cells ($\sim 10^6$). This method provides an estimate of the average telomere length per sample and produces subtelomeric polymorphism	[52, 128]
qPCR	The qPCR technique was first introduced by RM Cawthon in 2002. The method involve detection of telomeric DNA with fluorescent signals (T) using partially mismatched primers in a 96-well format on real time-quantitative PCR (qPCR) platform. The measurement of the telomeric DNA is normalized with a single-copy housekeeping gene (S) that is amplified in same sample in a different plate and T/S ratio (measure of relative TL) is computed	In qPCR technique, relative TL can be assessed within a short time and small amount of DNA (20 ng per reaction) is required. This method is widely used in epidemiological studies that involve large number of samples	Studies have shown that there is wide range of CVs (2–28%) for measurement of TL by qPCR which suggests that repeatability is a concern with the qPCR technique. Proper optimization of qPCR conditions is required to reduce variability	[129, 130, 133–135]
Monochrome multiplex qPCR (MMqPCR)	It was established by the same person as qPCR. This is an improved version of the qPCR method in which both telomeric DNA and single-copy gene are amplified in same well of a plate	In this method there is less variability compared with monoplex qPCR and has lesser sample requirement	Just like the qPCR proper optimization of experimental conditions is required	[130]
Flow-fluorescence *in situ* hybridization (FISH) assay	Telomere length analysis by FISH is based on the specific labeling of telomeres with fluorescent peptide nucleic acid (PNA) oligonucleotide probes	Increased sensitivity and specificity. It has ability to measure telomere length at the single-cell level	This method requires expensive equipment which may not be found in many laboratories and it is not possible to assess TL in tissues (histological samples) and stored samples. The assay typically requires cells to be at replicating stage to analyze chromosomes in metaphases rather than interphase cells	[131]
Single telomere length analysis (STELA)	It is a ligation PCR-based method	No specialized equipment is required and it requires very limited starting material	The single telomere length analysis method is also labor intensive and is again not appropriate for the analysis of large number of samples	[132]

Table 3.
Different methods for measurement of telomere length.

These modifications may include: rising of the rate of analysis, substitution of the radioactive label by nonlabeled compounds, the decrease in the amount of side products, and so on. A number of modifications still make it possible to detect telomerase activity within a single cell [84]. The TRAP consists of three main stages: primer elongation, amplification of telomerase-synthesized DNA, and finally, the detection step. In the elongation step, telomeric repeats are added to the telomere-imitating oligonucleotide (TS) by telomerase found in the cell extract. Next, PCR-amplification of telomerase synthesized DNA is carried out using definite primers (telomere-imitating and reverse primers). At this stage, different labels such as radioactive or fluorescent labels can be incorporated into the synthesized DNA. This step is then followed by detection through separation of PCR products by gel electrophoresis and imaging) [83]. The original TRAP assay has several drawbacks. Initially, the CX oligonucleotide, which complementarily overlaps with TS for several base pairs (bp), is used in the amplification of PCR products which results in the primer dimer formation as a result of the interaction between primers and products [83]. The use of optimal ACX primer with the noncomplementary TS end can lead to appearance of background signal during the analysis of concentrated tissue extracts from tumor [85]. The use of an oligonucleotide TSG4 which can also be added to the TRAP mixture in order to evaluate the effect of duplex-stabilizing inhibitors. This oligonucleotide does not require the synthesis of several repeats by telomerase before the inhibitor begins its action [86]. Various nucleotides used in TRAP assay were discussed in more detail [87]. In addition, when PCR is used for signal amplification, the PCR inhibitors contained in the specimen can alter the results of telomerase activity detection [83]. Previously, in the TRAP method, PCR products were detected in polyacrylamide gel (PAGE) with respect to the radioactive label used, it can be introduced using a radioactively labeled primer or incorporated into the DNA during the preparation of the PCR reaction. This method allows performing a qualitative assessment of the activity and processivity of telomerase in cells and tissue extracts [83]. In the second stage of TRAP, the PCR product allows to obtain an amount of DNA sufficient for gel staining, for instance, use of ethidium bromide [88], silver nitrite [89] and SYBR Green [90] and its analogs (which has sensitivity equal to that of radioactive label [91], while mutagenicity is considerably higher than when ethidium bromide is used) (**Tables 1** and **2**).

4.3 Methods used for measurement of telomere length (TL)

Several researchers have shown interest in measuring telomere length (TL) accurately and efficiently so as to understand both the fundamental biology of telomere maintenance as well as factors which contributes significantly to accelerated TL attrition. Tarik et al. [126, 127] have described different techniques which were developed for telomere length measurement (**Table 3**).

5. Conclusions

Telomerase is a ribonucleoprotein complex, composed of a reverse transcriptase enzyme catalytic subunit and a long non-coding RNA that contains the template sequence for telomere synthesis and is required for linear chromosome maintenance in most eukaryotes. Telomerase is a high-molecular weight RNP complex that consists of two major components: TERC and a TERT. It was found that in humans; only the TERT and TERC components of the telomerase are required for its activity *in vitro*, even though some proteins which have regulatory function are also essential for the catalytic function of telomerase *in vivo*. It was estimated that about 32

different proteins are involved with human telomerase *in vivo* which maintain its functionality and only some of these proteins are phylogenetically preserved. The molecular chaperones Hsp90 and p23, which directly associate with hTERT, are present in rabbit reticulocyte lysates and are necessary for telomerase activity. Studies suggest that in *Tetrahymena* the proteins p80 and p95 are not core telomerase components and may be separate ribonucleoproteins that copurified non-specifically with telomerase. Telomerase remains active during early embryonic development but expression declines after the blastocyst stage and can no longer be detected in neonatal somatic cells.

Until recently, the activity of telomerase was thought to be controlled by limiting access to the telomeres but it is regulated by some protein complexes such as the shelterin complexes. Nevertheless, the cumulative information given in this chapter show that the events involved in telomerase recruitment and its activation are separate. Although, the organizational biology and biochemistry responsible for the process of telomerase activation is still unknown which could be an important focus area in future research. Telomerase activity is highly expressed in embryonic germ cells, testes, ovaries and in some cancer cells but its activity is low or absent in somatic cells. The expression of telomerase activity in cells indicates replicative capability of that cell and this involves several factors which regulate the telomerase activity. Towards understanding the biology of telomere, several methods have been designed to measure the telomerase activity and the telomere length. TRAP was the initial method deployed for the measurement of telomerase where the amplified product is detected using gel electrophoresis. There are several other modifications to the original TRAP which has more advantages, such as the qPCR amplification method which uses less concentration of primers and permits quantitative determination of synthesized DNA. However, proper optimization of qPCR conditions is required to achieve reproducibility of this method. There are various methods which have been established for measurement of telomere length (TL) and these includes: (i) terminal restriction fragment (TRF) analysis (the gold standard), (ii) flow-FISH cytometry of cells following hybridization with fluorescent peptide nucleic acid (PNA) probes, (iii) quantitative fluorescence in situ hybridization (FISH) with fluorescent telomere PNA probes and (iv) qPCR assay. Monochrome multiplex qPCR (MMqPCR) was also established which is an improved version of the qPCR method in which both telomeric DNA and single-copy gene are amplified in a same well of a plate which require lesser sample and shows less variability. Studies have shown that there is wide range of CVs (2–28%) for measurement of TL by qPCR which suggests that repeatability is a concern with the qPCR technique. Therefore, proper optimization of qPCR protocols is required to reduce variability in the results.

Apart from the TRF assay all other methods have the problem of generating a relative measure of TL. While the qPCR technique has more advantage where it requires small amounts of DNA, less time consuming and can easily be performed in high-throughput format which makes it possible to analyze large epidemiological samples.

Conflict of interest

The authors have declared that there is no conflict of interest.

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