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. These interact with telomere terminal repeat (TERT) to form telomere-telomere interactions (TTI) and telomere-telomere interactions (TTI). The TTI interactions help to maintain telomere length and prevent telomere dysfunction. 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](image-url)

**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
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

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].
ribonucleoprotein that was copurified nonspecifically with telomerase [40]. Never-
theless, 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 mam-
nalian homolog of p80, and is involved with telomerase activity. [39]. TEP1 con-
ists 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 telo-
mere 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 asso-
ciation with hTERT in mammalian cells and in \textit{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 chro-
mosome 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 suffi-
cient cell divisions to cause critical shortening of the telomeres. Some cells, includ-
ing 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 \textit{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 division [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 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 | This modification is safer and easier to detect telomerase product | | [99] |
| 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 | 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 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 | 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 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 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] |
| 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 | | |
| 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 |
|------------|-----------------------------------------------|-------------------------|---------------------|------------|
| 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. The second cannot. Because of this, the probability of binding another DNA target to the same nanoparticle is reduced. The electroactive complex \([\text{Ru(NH}_3)_6]^3+\), which is capable of binding to negatively charged DNA chains due to electrostatic interactions, is used for detection. | 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 | [83, 119] |
| Optical biosensor 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 extinction 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 and a complementary DNA region on its 5'-end is used for detection. 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 sensitivity for clinical samples is low. | 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 | [118] |
| Quantum dot telomerase detection | This method involves the use of a quantum dot nanoparticle (fluorescing by absorbing a quantum with a wavelength of \(\lambda_1\) (400 nm) and emitting a quantum with a wavelength of \(\lambda_1'\) (560 nm). The method allows the determination of the telomerase activity in cell line extracts even though, it has not been tested on clinical materials. The sensitivity is sixty (65) T293 cells. 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, thus, reducing the cost of analysis. | 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] |
| Quantum dot telomerase activity detection | This method involves the use of a sensor chip (a transistor comprising antibodies coated silicon chip) to determine telomerase activity in cell line extracts even though, it has not been tested on clinical materials. The sensitivity is sixty (65) T293 cells. 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, thus, reducing the cost of analysis. | 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 | [121, 122] |

**Telomerase and Non-Telomerase Mechanisms of Telomere Maintenance**
| 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 (~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 involves 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 nonspecifically 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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References

[1] Armstrong CA, Tomita K. Fundamental mechanisms of telomerase action in yeasts and mammals: Understanding telomeres and telomerase in cancer cells. Open Biology. 2017;7:160338

[2] Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. Cell. 1985;43:405-413

[3] Burgstaller JP, Brem G. Aging of cloned animals: A mini-review. Gerontology. 2017;63:417-425

[4] Tian X, Doerig K, Park R, Can Ran Qin A, Hwang C, Neary A, et al. Evolution of telomere maintenance and tumour suppressor mechanisms across mammals. Philosophical Transactions of the Royal Society B. 2018;373:20160443

[5] Harrington L. Making the most of a little: Dosage effects in eukaryotic telomere length maintenance. Chromosome Research. 2005;13:493-504

[6] Hiyama E, Hiyama K. Telomere and telomerase in stem cells. British Journal of Cancer. 2007;96:1020-1024

[7] Liu Y, Kha H, Ungrin M, Robinson MO, Harrington L. Preferential maintenance of critically short telomeres in mammalian cells heterozygous for mTert. Proceedings of the National Academy of Sciences of the United States of America. 2002;99:3597-3602

[8] Autexier C, Lue NF. The structure and function of telomerase reverse transcriptase. Annual Review of Biochemistry. 2006;75:493-517

[9] Greider CW. Telomere length regulation. Annual Review of Biochemistry. 1996;65:337-365

[10] Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. Specific association of human telomerase activity with immortal cells and cancer. Science. 1994;266(5193):2011-2015

[11] Pardue ML, DeBaryshe PG. Retrotransposons provide an evolutionarily robust nontelomerase mechanism to maintain telomeres. Annual Review of Genetics. 2003;37:485-511

[12] Giardini MA, Segatto M, da Silva MS, Nunes VS, Cano MIN. Telomere and telomerase biology. Progress in Molecular Biology and Translational Science. 2014;125:1-40

[13] Chen Q, Ijpma A, Greider CW. Two survivor pathways that allow growth in the absence of telomerase are generated by distinct telomere recombination events. Molecular and Cellular Biology. 2001;21:1819-1827

[14] Lundblad V, Blackburn EH. An alternative pathway for yeast telomere maintenance rescues est1-senescence. Cell. 1993;73:347-360

[15] Tomas-Loba A, Flores I, Fernandez-Marcos PJ, et al. Telomerase reverse transcriptase delays aging in cancer-resistant mice. Cell. 2008;135:609-622

[16] Blackburn EH. Telomeres and their synthesis. Science. 1990;249:489-490

[17] Wu RA, Upton HE, Vogan JM, Collins K. Telomerase mechanism of telomere synthesis. Annual Review of Biochemistry. 2017;86:439-460

[18] Min J, Wright WE, Jerry WS. Alternative lengthening of telomeres mediated by mitotic DNA synthesis engages break-induced replication processes. Molecular and Cellular Biology. 2017;37(20):e00226-e00217
[19] Sandin S, Rhodes D. Telomerase structure. Current Opinion in Structural Biology. 2014;25:104-110

[20] Chen JL, Greider CW. Functional analysis of the pseudoknot structure in human telomerase RNA. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(23):8080-8085; discussion 8077-9. DOI: 10.1073/pnas.0502259102

[21] Chen JL, Greider CW. An emerging consensus for telomerase RNA structure. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(46):16391

[22] Chen JL, Greider CW. Telomerase RNA structure and function: Implications for dyskeratosis congenita. Trends in Biochemical Sciences. 2004;29(4):183-192

[23] Chen JL, Greider CW. Template boundary definition in mammalian telomerase. Genes & Development. 2003;17(22):2747-2752

[24] Chen JL, Greider CW. Determinants in mammalian telomerase RNA that mediate enzyme processivity and cross-species incompatibility. The EMBO Journal. 2003;22(2):304-314

[25] Chen JL, Opperman KK, Greider CW. A critical stem-loop structure in the CR4-CR5 domain of mammalian telomerase RNA. Nucleic Acids Research. 2002;30(2):592-597

[26] Chen JL, Blasco MA, Greider CW. Secondary structure of vertebrate telomerase RNA. Cell. 2000;100(5):503-514

[27] Harrington L. Biochemical aspects of telomerase function. Cancer Letters. 2003;194:139-154

[28] Cohen SB, Graham ME, Lovrecz GO, Bache N, Robinson PJ, Reddel RR. Protein composition of catalytically active human telomerase from immortal cells. Science. 2007;315:1850-1853

[29] Collins K. The biogenesis and regulation of telomerase holoenzymes. Nature Reviews. Molecular Cell Biology. 2006;7:484-494

[30] Lingner J, Cech TR, Hughes TR, Lundblad V. Three ever shorter telomere (EST) genes are dispensable for in vitro yeast telomerase activity. Proceedings of the National Academy of Sciences of the United States of America. 1997;94:11190-11195

[31] Wong JMY, Collins K. Telomere maintenance and disease. Lancet. 2003;362:983-988

[32] Fu D, Collins K. Purification of human telomerase complexes identifies factors involved in telomerase biogenesis and telomere length regulation. Molecular Cell. 2007;28:773-785

[33] Smogorzewska A, de Lange T. Regulation of telomerase by telomeric proteins. Annual Review of Biochemistry. 2004;73:177-208

[34] Nandakumar J, Cech TR. Finding the end: Recruitment of telomerase to telomeres. Nature Reviews. Molecular Cell Biology. 2013;14:69-82

[35] Gilson E, Geli V. How telomeres are replicated. Nature Reviews. Molecular Cell Biology. 2007;8:825-838

[36] Londono-Vallejo JA, Wellinger RJ. Telomeres and telomerase dance to the rhythm of the cell cycle. Trends in Biochemical Sciences. 2012;37:391-399
Telomerase and Non-Telomerase Mechanisms of Telomere Maintenance

[37] Stewart JA, Wang F, Chaiken MF, et al. Human CST promotes telomere duplex replication and general replication restart after fork stalling. The EMBO Journal. 2012;31:3537-3549

[38] Watson JD. Origin of concatemeric T7 DNA. Nature: New Biology. 1972;239:197-201

[39] Cong YS, Wright WE, Shay JW. Human telomerase and its regulation. Microbiology and Molecular Biology Reviews: MMBR. 2002;66(3):407-425

[40] Mason JM, Frydrychova RC, Biessmann H. Drosophila telomeres: An exception providing new insights. BioEssays. 2008;30:25-37

[41] Liu Y, Snow BE, Hande MP, et al. The telomerase reverse transcriptase is limiting and necessary for telomerase function in vivo. Current Biology. 2000;10:1459-1462

[42] Szostak JW, Blackburn EH. Cloning yeast telomeres on linear plasmid vectors. Cell. 1982;29:245-255

[43] Zakian VA. Telomeres: Beginning to understand the end. Science. 1995;270:1601-1607

[44] Hiyama K, Hirai Y, Kyoizumi S, Akiyama M, Hiyama E, Piatyszek MA, et al. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. Journal of Immunology. 1995;155:3711-3715

[45] Jafri MA, Ansari SA, Alqahtani MH, Shay JW. Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies. Genome Medicine. 2016;8:69

[46] Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. European Journal of Cancer. 1997;33:787-791

[47] Bodnar AG et al. Extension of lifespan by introduction of telomerase into normal human cells. Science. 1998;279:349-352

[48] de Lange T, Shiue L, Myers RM, Cox DR, Naylor SL, Killery AM, et al. Structure and variability of human chromosome ends. Molecular and Cellular Biology. 1990;10:518-527

[49] Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC. Telomere reduction in human colorectal carcinoma and with ageing. Nature. 1990;346:866-868

[50] Xu L, Blackburn EH. Human cancer cells harbor T-stumps, a distinct class of extremely short telomeres. Molecular Cell. 2007;28:315-327

[51] Teixeira MT, Arneric M, Sperisen P, Lingner J. Telomere length homeostasis is achieved via a switch between telomerase-extendible and nonexpendable states. Cell. 2004;117:323-335

[52] Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature. 1990;345(6274):458-460

[53] Wright WE, Piatyszek MA, Rainey WE, Byrd W, Shay JW. Telomerase activity in human germline and embryonic tissues and cells. Developmental Genetics. 1996;18:173-179

[54] Gilchrist GC, Kurjanowicz P, Mereilles FV, King WA, LaMarre J. Telomere length and telomerase activity in bovine pre-implantation embryos in vitro. Reproduction in Domestic Animals. 2015;50:58-67. DOI: 10.1111/rdm.12449

[55] Turner S, Wong HP, Rai J, Hartshorne GM. Telomere lengths in human oocytes, cleavage stage embryos
and blastocysts. Molecular Human Reproduction. 2010;16:685-694

[56] Ramirez RD, Wright WE, Shay JW, Taylor RS. Telomerase activity concentrates in the mitotically active segments of human hair follicles. The Journal of Investigative Dermatology. 1997;108:113-117

[57] Greenwood MJ, Lansdorp PM. Telomeres, telomerase, and hematopoietic stem cell biology. Archives of Medical Research. 2003;34:489-495

[58] Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Experimental Cell Research. 1961;25:585-621

[59] Djojosubroto MW, Choi YS, Lee HW, Rudolph KL. Telomeres and telomerase in aging, regeneration and cancer. Molecules and Cells. 2003;15:164-175

[60] Schaetzlein S, Rudolph KL. Telomere length regulation during cloning, embryogenesis and ageing. Reproduction, Fertility, and Development. 2005;17:85-96

[61] Sharpless NE, DePinho RA. Telomeres, stem cells, senescence, and cancer. The Journal of Clinical Investigation. 2004;113:160-168

[62] Shay JW, Wright WE. Senescence and immortalization: Role of telomeres and telomerase. Carcinogenesis. 2005;26:867-874

[63] Blackburn EH. Cell biology: Shaggy mouse tales. Nature. 2005;436:922-923

[64] Hou M, Wang X, Popov N, et al. The histone deacetylase inhibitor trichostatin A derepresses the telomerase reverse transcriptase (hTERT) gene in human cells. Experimental Cell Research. 2002;274:25-34

[65] Xu D, Popov N, Hou M, et al. Switch from Myc/Max to Mad1/Max binding and decrease in histone acetylation at the telomerase reverse transcriptase promoter during differentiation of HL60 cells. Proceedings of the National Academy of Sciences of the United States of America. 2001;98:3826-3831

[66] Gladych M, Wojtyla A, Rubis B. Human telomerase expression regulation. Biochemistry and Cell Biology. 2011;89:359-376

[67] Blasco MA. Telomeres and human disease: Ageing, cancer and beyond. Nature Reviews. Genetics. 2005;6:611-622

[68] Cesare AJ, Reddel RR. Alternative lengthening of telomeres: Models, mechanisms and implications. Nature Reviews. Genetics. 2010;11:319-330

[69] Ulaner GA, Hu JF, Vu TH, Giudice LC, Hoffman AR. Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. Cancer Research. 1998;58:4168-4172

[70] Blasco MA, Rizen M, Greider CW, Hanahan D. Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. Nature Genetics. 1996;12:200-204

[71] Lu W, Zhang Y, Liu D, Songyang Z, Wan M. Telomeres-structure, function, and regulation. Experimental Cell Research. 2013;319:133-141

[72] Rahman R, Latonen L, Wiman KG. hTERT antagonizes p53-induced apoptosis independently of telomerase activity. Oncogene. 2005;24:1320-1327

[73] Henson JD, Neumann AA, Yeager TR, Reddel RR. Alternative lengthening of telomeres in mammalian cells. Oncogene. 2002;21:598-610
Telomerase and Non-Telomerase Mechanisms of Telomere Maintenance

[74] Teng SC, Zakian VA. Telomere-telomere recombination is an efficient bypass pathway for telomere maintenance in *Saccharomyces cerevisiae*. Molecular and Cellular Biology. 1999;19:8083-8093

[75] Reddel RR. Alternative lengthening of telomeres, telomerase, and cancer. Cancer Letters. 2003;194:155-162

[76] Counter CM, Avilion AA, LeFeuvre CE, et al. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. The EMBO Journal. 1992;11:1921-1929

[77] Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. The EMBO Journal. 1995;14:4240-4248

[78] Pardue ML, DeBaryshe PG. Drosophila telomeres: A variation on the telomerase theme. Fly (Austin). 2008;2:101-110

[79] Dreesen O, Cross GA. Telomerase-independent stabilization of short telomeres in *Trypanosoma brucei*. Molecular and Cellular Biology. 2006;26:4911-4919

[80] Dreesen O, Cross GA. Consequences of telomere shortening at an active VSG expression site in telomerase-deficient *Trypanosoma brucei*. Eukaryotic Cell. 2006;5:2114-2119

[81] Dreesen O, Li B, Cross GA. Telomere structure and function in trypanosomes: A proposal. Nature Reviews. Microbiology. 2007;5:70-75

[82] Hovel-Miner GA, Boothroyd CE, Muguier M, Dreesen O, Cross GA, Papavasiliou FN. Telomere length affects the frequency and mechanism of antigenic variation in *Trypanosoma brucei*. PLoS Pathogens. 2012;8:e1002900

[83] Skvortsov DA, Zvereva ME, Shpanchenko OV, Donsotva OA. Assays for detection of telomerase activity. Acta Naturae. 2011;3(1):48-68

[84] Wright WE, Shay JW, Piatyszek MA. Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. Nucleic Acids Research. 1995;23(18):3794-3795

[85] Skvortsov DA, Gasparyan NM, Rubtsova MP, et al. Telomerase as a potential marker for early diagnosing cervical carcinoma. Dokl Biochem Biophys. 2006;408:158-160

[86] Gomez D, Mergny JL, Riou JF. Detection of telomerase inhibitors based on G-quadruplex ligands by a modified telomeric repeat amplification protocol assay. Cancer Research. 2002;62(12):3365-3368

[87] Saldanha SN, Andrews LG, Tollefsbol TO. Analysis of telomerase activity and detection of its catalytic subunit, hTERT. Analytical Biochemistry. 2003;315(1):1-21

[88] Gan Y, Lu J, Johnson A, Wientjes MG, Schuller DE, Au JL. A quantitative assay of telomerase activity. Pharmaceutical Research. 2001;18(4):488-493

[89] Dalla Torre CA, Maciel RM, Pinheiro NA, Andrade JA, De Toledo SR, Villa LL, et al. TRAP-silver staining, a highly sensitive assay for measuring telomerase activity in tumor tissue and cell lines. Brazilian Journal of Medical and Biological Research. 2002;35(1):65-68

[90] Holt SE, Norton JC, Wright WE, Shay JW. Comparison of the telomeric repeat amplification protocol (TRAP) to
the new TRAP-eze telomerase detection kit. Methods in Cell Science. 1996;18:237-248

[91] Skvortsov DA, Zvereva ME, Pavlova LS, Petrenko AA, Kisseljov FL, Dontsova OA. Vestn. MSU. 2010;65(3):165-169

[92] Gollahon LS, Holt SE. Alternative methods of extracting telomerase activity from human tumor samples. Cancer Letters. 2000;159(2):141-149

[93] Uehara H, Nardone G, Nazarenko I, Hohman RJ. Detection of telomerase activity utilizing energy transfer primers: Comparison with gel- and ELISA-based detection. BioTechniques. 1999;26(3):552-558

[94] Szatmari I, Tokes S, Dunn CB, Bardos TJ, Aradi J. Modified telomeric repeat amplification protocol: A quantitative radioactive assay for telomerase without using electrophoresis. Analytical Biochemistry. 2000;282(1):80-88

[95] Szatmari I, Aradi J. Telomeric repeat amplification, without shortening or lengthening of the telomerase products: A method to analyze the processivity of telomerase enzyme. Nucleic Acids Research. 2001;29(2):E3. DOI: 10.1093/nar/29.2.e3

[96] Bazin H, Preaudat M, Trinquet E, Mathis G. Homogeneous time resolved fluorescence resonance energy transfer using rare earth cryptates as a tool for probing molecular interactions in biology. Spectrochimica Acta. Part A, Molecular and Biomolecular Spectroscopy. 2001;57(11):2197-2211

[97] Gabourdes M, Bourgine V, Mathis G, Bazin H, Alpha-Bazin B. A homogeneous time-resolved fluorescence detection of telomerase activity. Analytical Biochemistry. 2004;333(1):105-113

[98] Savoysky E, Akamatsu K, Tsuchiya M, Yamazaki T. Detection of telomerase activity by combination of TRAP method and scintillation proximity assay (SPA). Nucleic Acids Research. 1996;24(6):1175-1176

[99] Hirose M, Abe-Hashimoto J, Ogura K, Tahara H, Ide T, Yoshimura TJ. A rapid, useful and quantitative method to measure telomerase activity by hybridization protection assay connected with a telomeric repeat amplification protocol. Journal of Cancer Research and Clinical Oncology. 1997;123(6):337-344

[100] Mayfield MP, Shah T, Flannigan GM, Hamilton Stewart PA, Bibby MC. Telomerase activity in malignant and benign bladder conditions. International Journal of Molecular Medicine. 1998;1(5):835-840

[101] Hoos A, Hepp HH, Kaul S, Ahlert T, Bastert G, Wallwiener D. Telomerase activity correlates with tumor aggressiveness and reflects therapy effect in breast cancer. International Journal of Cancer. 1998;79(1):8-12

[102] Chen L, Huang J, Meng F, Zhou N. Distinguishing tumor cells via analyzing intracellular telomerase activity. Analytical Sciences. 2010;26(5):535-538

[103] Hou M, Xu D, Bjorkholm M, Gruber A. Real-time quantitative telomeric repeat amplification protocol assay for the detection of telomerase activity. Clinical Chemistry. 2001;47(3):519-524

[104] Elmore LW, Forsythe HL, Ferreira-Gonzalez A, Garrett CT, Clark GM, Holt SE. Real-time quantitative analysis of telomerase activity in breast tumor specimens using a highly specific and sensitive fluorescent-based assay. Diagnostic Molecular Pathology. 2002;11(3):177-185
[105] Heller-Uszynska K, Kilian A. Microarray TRAP: A high-throughput assay to quantitate telomerase activity. Biochemical and Biophysical Research Communications. Oct 2004;323(2):465-472

[106] Ohyashiki K, Ohyashiki JH, Nishimaki J, Toyama K, Ebihara Y, Kato H, et al. Cytological detection of telomerase activity using an in situ telomeric repeat amplification protocol assay. Cancer Research. 1997;57(11):2100-2103

[107] Yahata N, Ohyashiki K, Ohyashiki JH, Iwama H, Hayashi S, Ando K, et al. Telomerase activity in lung cancer cells obtained from bronchial washings. Journal of the National Cancer Institute. 1998;90(9):684-690

[108] Ohyashiki K, Yahata N, Ohyashiki JH, Iwama H, Hayashi S, Ando K, et al. A combination of semiquantitative telomerase assay and in-cell telomerase activity measurement using exfoliated urothelial cells for the detection of urothelial neoplasia. Cancer. 1998;83(12):2554-2560

[109] Dejmek A, Yahata N, Ohyashiki K, Kakhiana M, Hirano T, Kawate N, et al. Correlation between morphology and telomerase activity in cells from exfoliative lung cytologic specimens. Cancer. 2000;90(2):117-125

[110] Dejmek A, Yahata N, Ohyashiki K, Ebihara Y, Kakhiana M, Hirano T, et al. In situ telomerase activity in pleural effusions: A promising marker for malignancy. Cytopathology. 2001;24(1):11-15

[111] Youssef N, Paradis V, Ferlicot S, Bedossa P. In situ detection of telomerase enzymatic activity in human hepatocellular carcinogenesis. The Journal of Pathology. 2001;194(4):459-465

[112] Hirose M, Abe-Hashimoto J, Tahara H, Ide T, Yoshimura T. New method to measure telomerase activity by transcription-mediated amplification and hybridization protection assay. Clinical Chemistry. 1998;44:2446-2452

[113] Blackburn EH, Greider CW, Henderson E, Lee MS, Shampay J, Shippen-Lentz D. Recognition and elongation of telomeres by telomerase. Genome. 1989;31(2):553-560

[114] Maesawa C, Inaba T, Sato H, Iijima S, Ishida K, Terashima M, et al. A rapid biosensor chip assay for measuring of telomerase activity using surface plasmon resonance. Nucleic Acids Research. 2003;31:e4

[115] Rad’ko SP, Voronina SA, Gromov AV, Gnedenko OV, Bodoev NV, Ivanov AS, et al. Use of oligonucleotides conjugated to gold nanoparticles and streptavidin for amplification of optical biosensor signal during detection of telomeric repeats. Bulletin of Experimental Biology and Medicine. 2009;147(6):746-749

[116] Grimm J, Perez JM, Josephson L, Weissleder R. Novel nanosensors for rapid analysis of telomerase activity. Cancer Research. 2004;64:639-643

[117] Pavlov V, Willner I, Dishon A, Kotler M. Amplified detection of telomerase activity using electrochemical and quartz crystal microbalance measurements. Biosensors & Bioelectronics. 2004;20(5):1011-1021

[118] Li Y, Liu B, Li X, Wei Q. Highly sensitive electrochemical detection of human telomerase activity based on barcode method. Biosensors & Bioelectronics. 2010;25(11):2543-2547

[119] Schmidt PM, Matthes E, Scheller FW, Bientert M, Lehmann C, Ehrlich A, et al. Real-time determination of telomerase activity in cell extracts
using an optical biosensor. Biological Chemistry. 2002;383:1659-1666

[120] Patolsky F, Gill R, Weizmann Y, Mokari T, Banin U, Willner I. Lighting-up the dynamics of telomerization and DNA replication by CdSe-ZnS quantum dots. Journal of the American Chemical Society. 2003;125(46):13918-13919

[121] Zheng G, Patolsky F, Cui Y, Wang WU, Lieber CM. Multiplexed electrical detection of cancer markers with nanowire sensor arrays. Nature Biotechnology. 2005;23(10):1294-1301

[122] Sharon E, Freeman R, Riskin M, Gil N, Tzfat Y, Willner I. Optical, electrical and surface plasmon resonance methods for detecting telomerase activity. Analytical Chemistry. 2010;82:8390-8397

[123] Xu SQ, He M, Yu HP, Wang XY, Tan XL, Lu B, et al. Bioluminescent method for detecting telomerase activity. Clinical Chemistry. 2002;48(7):1016-1020

[124] Zhou X, Xing D, Zhu D, Jia L. Magnetic bead and nanoparticle based electrochemiluminescence amplification assay for direct and sensitive measuring of telomerase activity. Analytical Chemistry. 2009;81(1):255-261

[125] Wu JY, Stone MD, Zhuang X. A single-molecule assay for telomerase structure-function analysis. Nucleic Acids Research. 2010;38(3):e16. DOI: 10.1093/nar/gkp1033

[126] Mohamad T, Ramakrishnan L, Sachdev HS, Tandon N, Roy A, Bhargava SK, et al. Validation of quantitative polymerase chain reaction with southern blot method for telomere length analysis. Future Science OA. 2018;4(4):FSO282

[127] O’Callaghan NJ, Fenech M. A quantitative PCR method for measuring absolute telomere length. Biological Procedures Online. 2011;13:3

[128] Kimura M, Stone RC, Hunt SC, et al. Measurement of telomere length by the southern blot analysis of terminal restriction fragment lengths. Nature Protocols. 2010;5(9):1596-1607

[129] Cawthon RM. Telomere measurement by quantitative PCR. Nucleic Acids Research. 2002;30(10):e47.26

[130] Cawthon RM. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. Nucleic Acids Research. 2009;37:e21. Paper describing previous procedure with full details of multiplex qPCR method

[131] Hultdin M, Gronlund E, Norrback K, Eriksson-Lindström E, Just T, Roos G. Telomere analysis by fluorescence in situ hybridization and flow cytometry. Nucleic Acids Research. 1998;26(16):3651-3656

[132] Baird DM, Rowson J, Wynford-Thomas D, Kipling D. Extensive allelic variation and ultrashort telomeres in senescent human cells. Nature Genetics. 2003;33(2):203-207

[133] De Vivo I, Prescottn J, Wong JY, Kraft P, Hankinson SE, Hunter DJ. A prospective study of relative telomere length and postmenopausal breast cancer risk. Cancer Epidemiology, Biomarkers & Prevention. 2009;18(4):1152-1156. Paper describing previous use of qPCR method and observed higher levels of CVs

[134] Shen J, Terry MB, Gurvich I, Liao Y, Senie RT, Santella RM. Short telomere length and breast cancer risk: A study in sister sets. Cancer Research. 2007;67(11):5538-5544. Paper describing previous use of qPCR method and observed higher levels of CVs
[135] Hsieh AYY, Saberi S, Ajay kumar A, et al. Optimization of a relative telomere length assay by monochromatic multiplex real-time quantitative PCR on the light cycler 480: Sources of variability and quality control considerations. The Journal of Molecular Diagnostics. 2016;18(3):425-437