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# Telomeres and Telomerase

*Methods and Protocols*

*Edited by*

**John A. Double**

**Michael J. Thompson**

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## **Telomeres and Telomerase**

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METHODS IN MOLECULAR BIOLOGY™

# Telomeres and Telomerase

*Methods and Protocols*

Edited by

**John A. Double**

and

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## Preface

The fundamental problem that dividing cells have to overcome is that of end-replication. Chromosomes shorten by many bases during DNA replication and so this presents a major hurdle that a cell has to overcome both to enable it to proliferate and for the larger organism to survive and reproduce. The enzyme telomerase provides a mechanism to ensure chromosome stability in both normal and neoplastic cells. The demonstration of telomerase expression in a majority of tumors and the realization of the potential role of telomerase in aging has opened up the potential for telomerase to be used as a target for therapeutic intervention.

There is therefore great interest in the expression and activity of telomerase in a wide range of biological disciplines. *Telomeres and Telomerase: Methods and Protocols* has been produced as a tool for the many researchers in different areas of cell biology who are interested in following research in the area of telomerase and telomere maintenance, either in the area of fundamental mechanisms or perhaps in the area of more applied drug discovery work.

*Telomeres and Telomerase: Methods and Protocols* covers a range of novel and essential telomerase assay protocols in step-by-step fashion allowing them to be easily repeated and applied by both experienced and telomerase-naïve researchers. The protocols allow a worker to identify and analyze telomeres, to determine telomerase expression at the RNA level. The chapters also describe various methods by which one can determine telomerase activity and detect potential modifiers of this activity. We trust this work will be found both informative and useful.

*John A. Double*  
*Michael J. Thompson*



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## Introduction to Telomeres and Telomerase

**Michael C. Bibby**

Telomeres are specialized nucleoproteins that have an important role in chromosome structure and function (*1*). The telomeric DNA together with its associated proteins protects the chromosome ends from degradation or aberrant recombination (*1,2*). In most eukaryotes telomeric DNA consists of tracts of simple, tandemly repeated sequences running 5' to 3' toward the distal ends of the chromosome. In humans the sequence TAGGG is repeated hundreds of thousands of times (*3–7*) but there can be large variations in the number of telomeric repeats between organisms; i.e., in ciliates there can be fewer than 50 nucleotides of repeated DNA, whereas some mouse strains have more than 100 kilobase (kb) repeats (*1,8*). Mammals show tissue-to-tissue variation in average telomere length (*6,7,9–11*) and within a single mammalian cell, length of telomeres varies between different chromosomes (*12,13*).

### 1. Cell Replication

Because of the mechanism of conventional DNA polymerases, the replication of DNA molecules can be predicted to result in the gradual shortening of the chromosome by the length of a terminal primer at each cell cycle (*1*). This predication is supported by the

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fact that average length of telomeres has been shown to shorten in a number of mammalian somatic cells as they proliferate *in vitro* and *in vivo*, whereas single-cell eukaryotes maintain telomeres at a relatively constant length (7,14). Mammalian germ cells also have the ability to maintain telomere length; therefore, a separate mechanism exists in these cells that is able to maintain telomere length. It is thought that in most eukaryotes the enzyme responsible for replication of the telomeres is telomerase. Although a number of alternative solutions to the end-replication problem exist in nature, for example, the retrotransposons utilized by dipterans like *Drosophila*, it appears that the telomerase solution is the most widespread and perhaps the oldest among eukaryotes (15).

Telomerase activity has been detected in G<sub>1</sub>, S and G<sub>2</sub>/M phases of the cell cycle (14) and similar levels of telomerase have been observed in phase-specific fractions of primary normal lymphocytes synchronized by drugs and separated by fluorescence-activated cell sorting (FACS) (16,17). Telomerase activity has been shown to decrease as cells differentiate in culture, and considerable information is being amassed on telomerase activity or RNA in relation to cellular proliferation, e.g., telomerase activity appears to be highest in the proliferating compartments of the seminiferous tubules of the testis as compared to the nondividing compartments (18). Interestingly, in the testis this activity is inversely correlated with telomere length (19), indicating that the relationship between telomerase and differentiation is not a straightforward one. Although there appears to be a relationship between telomerase expression and proliferation and differentiation, more specific links have not been identified.

## 2. Telomerase Structure

Telomerase is a specialized DNA polymerase that synthesizes telomeric repeats *de novo*. It consists of an RNA subunit that acts as the template for the synthesis of telomeric DNA, and this process is catalyzed by a protein component (20). Therefore because telomerase polymerizes DNA it is a true reverse transcriptase. The RNA component of telomerase was first characterized in ciliates (21,22).

The genes for the human (hTR) and mouse (*terc*) RNA components and for the human protein component (hTRT) have been cloned (23–27), and the catalytic protein subunits have also been identified in *Euplotes aediculatus* and *Saccharomyces cerevisiae* (28) as well as in *Schizosaccharomyces pombe* (25). Nakamura and Cech (15) point to some inconsistencies in terminology, as the gene and proteins have been called previously hTRT (25), hEST2 (29), TCS1 (30) and TP2 (31). The Genome Database (GDB) has approved the name hTERT for the human gene. As a first step in attempting to understand the factors that repress or activate hTR and *terc* expression, Zhao et al. (32) cloned the promoter regions of these human and mouse genes. Recent work has further resolved the functional domains of hTERT (33,34) and indicated its central role in determining telomerase activity (35,36) but not necessarily telomere maintenance or immortality (37). The important role of hTERT has identified it as an important target for drug development (38).

### 3. Senescence and Immortalization

Aging of normal cells is a result of their limited proliferative capacity. After attaining their finite life span, normal cells cease dividing and senesce. It appears that cells lacking telomerase progressively lose telomeres, resulting in senescence, and it has been suggested that the sequential shortening of telomeric DNA may be an important molecular timing mechanism (39). On the other hand, germ cells and immortal cell lines express telomerase and maintain telomere length through countless cell divisions. It has been shown for some time that the telomerase RNA component in ciliates is upregulated along with telomerase activity (40,41), and more recently it was shown that similarly, most mouse tissues have telomerase activity that roughly correlates with RNA expression levels (23). On the other hand, many human tissues appear to be telomerase negative, although there is evidence of RNA expression in a number of tissues (24) and stem cells. It is possible that the differences in detectable telomerase levels between mouse and human cells might provide an explanation for the relative ease by

which primary cultures of mouse fibroblasts undergo spontaneous immortalization compared to human primary cultures (42). The investigations into the role of telomerase in aging has taken one or two interesting turns lately. Firstly it has been shown that disruption of the function of telomerase by molecular genetic manipulations results in telomere shortening and cell death in cultured cell lines (24) but some cancer cell lines that are telomerase negative possess long or even hyperelongated telomeres (43). A further setback to the telomerase theory came from the studies of Blasco et al. (44), who by the development of a telomerase knockout mouse indicated that although telomerase appeared to be required for telomere length maintenance, it was not required for the establishment of immortalized cell lines from these mice. The mice survived and reproduced over six consecutive generations, indicating that neither telomere length nor telomerase was important for development or survival. However, in a follow up study the group performed a phenotypic analysis of each generation (45). They described progressive adverse effects of telomerase deficiency on the reproductive and hematopoietic systems. Late-generation animals exhibited defective spermatogenesis with increasing apoptosis and decreased proliferation in the testis, and bone marrow and spleen had impaired proliferative capacity. These effects accompanied substantial erosion of telomeres, as well as fusion and loss of chromosomes. The investigators concluded from their findings that maintenance of genomic integrity and long-term viability of high-renewal organ systems rely on telomerase and, hence, telomeres.

*In vitro* transcription and translation of hTERT when cosynthesized or mixed with the human telomerase RNA component (hTR), reconstitutes telomerase activity that exhibits enzymatic properties like those of the native enzyme (20), indicating that these are the two only essential components for activity. Because some normal human somatic cells do express hTR but have no detectable telomerase activity and nearly undetectable amounts of mRNA encoding hTERT, these investigators suggested that telomerase activity could be restored in these cells by transient expression of hTERT. Normal human diploid cells were transfected with an expression

plasmid encoding hTERT or a control vector, and the study demonstrated that only extracts from cells transfected with the hTERT plasmid possessed telomerase activity. Normal human cells with stable expression of introduced telomerase have been shown subsequently to exhibit an increased life span (46). These data are considered by some to provide direct evidence that telomere shortening controls cellular aging (18,47).

#### **4. Cancer**

Telomerase expression can be detected in the majority of human cancers (48) and there is ever-increasing literature on straightforward descriptions of the results of telomeric repeat amplification protocol (TRAP) assays (49) on a whole host of tumor types. As a result of this wealth of evidence for telomerase expression in malignancy, considerable discussion has been centered around the idea of its use in cancer diagnosis and staging (50–60). A number of studies now suggest that telomerase expression may be a marker of premalignant and malignant lesions, but there is also a note of caution as some studies point to individual cases where it is simply not a good marker (61), certain limitations are apparent (62), normal epithelial cells are positive (63–66) or activity does not correlate with important prognostic markers such as survival (67). Some work indicates that certain tumor types possess additional mechanisms by which they regulate telomere length (68).

The knockout mouse gene study described above (69) has also indicated the need for caution in interpreting the role of telomerase in malignancy. Cells from the fourth generation of these mice completely lacked telomere repeats, yet they could be immortalized in culture. To date, much of the available literature on human cancers and normal tissues describes TRAP assay data alone, and although this highly sensitive assay opened up the whole field, there are a number of pitfalls when it comes to applying it to biopsy material or surgical specimens. Also, the methodology for measuring telomere length is not standard across laboratories at present, methods must be optimized and protocols agreed upon before telomere length can

be evaluated as a predictor of prognosis. In the case of telomerase, it is clearly much more useful to be able to examine paraffin-embedded tissues for RNA expression or use *in situ* assays, which allow for careful scrutiny of different cell populations. As improvements in methodology occur, it is likely that reliable and simple tests for telomerase expression will be developed, thus allowing useful information to be acquired on a routine basis. Only then can the clinical usefulness of telomerase as a cancer marker be resolved.

## 5. Telomerase Inhibition

The differential expression of telomerase between malignancies and normal somatic tissues (70), as well as the suggestion that telomerase is essential for immortalization, introduced the possibility of enzyme inhibition as an exciting prospect for cancer therapy. Although at present the situation regarding the role of telomerase in senescence, immortalization and cancer, and in the maintenance of essential stem cell compartments is far from simple and the subject of much debate and many studies, the search for specific inhibitors should be encouraged. Because it is likely that a large number of human tumors have short telomeres and rely on telomerase for continued proliferation, there may well be an opportunity for relatively specific treatments in these malignancies.

In conclusion, over relatively few years there has been an enormous upsurge in the amount of research on this fascinating topic that has begun to dissect out the fundamental role of telomerase in cell growth and survival. Research in this area has benefited greatly from the development of sophisticated and specific assays, and the assay development itself has provided an exciting challenge to the scientists involved. For the field to continue to advance at this impressive rate and to enable us to take advantage of discoveries to develop therapeutic strategies, it is hoped that the field of telomerase research will continue to provide an interesting challenge. It is likely that the debate on the role of telomerase in somatic tissue and in aging will continue for some time and there is still useful work to be

done before the real potential of telomerase in cancer diagnosis and of antitelomerase strategies is fully evaluated.

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## Detection of Chromosome Ends by Telomere FISH

Harry Scherthan

### 1. Introduction

Vertebrate chromosomes end in a variable number of T<sub>2</sub>AG<sub>3</sub> repeats (**1**), which, jointly with associated proteins, are essential for chromosomal stability (for reviews, *see* refs. **2–4**). Telomeres have important roles in essential cellular processes like replication, malignant transformation, and cellular aging (*see* ref. **5–8**). Telomere repeats make up to 2–50 kb of DNA (**1,9,10**), which are complexed with TTAGGG repeat binding factor (TRF) proteins (**11,12**). At interphase, telomeres are dispersed throughout the nuclear lumen (**13,14**) and appear to be associated with the nuclear matrix (**15**). In most somatic cell types replicative shortening leads to an interchromosomal variation of terminal T<sub>2</sub>AG<sub>3</sub> repeats (**14,16,17**) which can be halted or restored by the DNA-dependent (RNP) polymerase telomerase (**8,18**). Furthermore, telomeres are key players in the chromosome-pairing process during meiosis. At the onset of meiotic prophase the scattered premeiotic (somatic) telomere distribution is altered such that telomeres attach to the inner nuclear membrane and then move along it to cluster in a limited nuclear

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envelope sector at the onset of zygotene (*see ref. 19*). This bouquet formation is thought to contribute to homolog recognition and pairing (e.g., *19–21*).

Because of the many vital functions telomeres perform in cellular processes, it may be of particular interest to detect and study telomeric regions in chromosomes and/or interphase nuclei of a given cell type. Numerous protocols and methods are available to delineate telomeric T<sub>2</sub>AG<sub>3</sub> repeats *in situ*. Among these are techniques like primed *in situ* (PRINS) labeling (*22,23*) or fluorescence *in situ* hybridization (FISH) with RNA-translated (*15*) or nick-translated, double-stranded DNA (dsDNA) repeat probes (*14,24*). Long oligonucleotides have proven effective telomere FISH probes (*see ref. 25*). FISH with short peptide nucleic acid (PNA) telomere probes yields detection efficiencies of nearly 100% and, in combination with digital fluorescence microscopy, allows for assessment of repeat amounts at individual chromosome ends (*7,16,17*). Outlined below are hapten-labeling and telomere FISH protocols for long oligonucleotide probes that have been successfully applied to study the distribution of telomeres in metaphase chromosomes and interphase nuclei (e.g., *19,26,27*) and usually render high hybridization efficiencies in metaphase and interphase chromosomes (*see Fig. 1*).

## **2. Materials**

### **2.1. General Lab Equipment for Molecular Cytogenetic Procedures.**

1. Water bath (preferably shaking).
2. Incubator.
3. Coplin jars with lids.
4. Glass slides.
5. Micropipettes.
6. Benchtop and microcentrifuge.
7. Forceps .
8. Phase-contrast and fluorescence microscope.

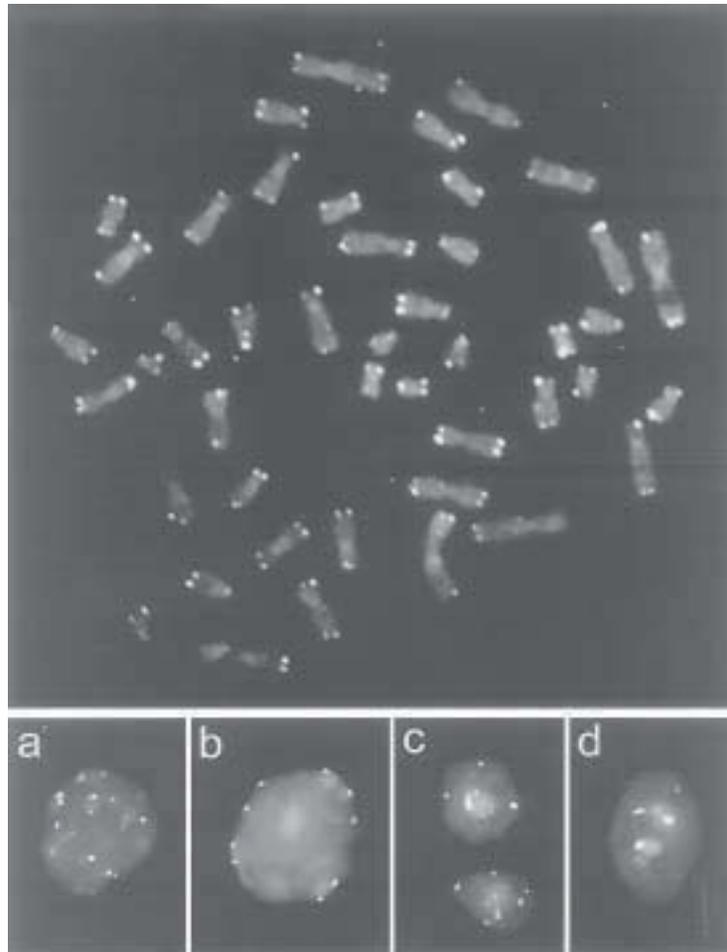


Fig.1. (Top panel) Telomere repeat detection (white signals) in human lymphocyte metaphase chromosomes by FISH with a biotinylated  $(T_2AG_3)_7/(C_3TA_2)_7$  oligonucleotide probe labels chromosome ends (color inverted image; chromosomal DNA was counterstained with PI). (Bottom panel) Telomere FISH of a mouse testis suspension discloses a differential distribution of telomeres (white signals) in nuclei of different cell types (DAPI, gray; color inverted images). **(a)** Nucleus of a spermatogonium with a scattered (somatic) telomere distribution. **(b)** Telomeres are exclusively located at the periphery of a pachytene spermatocyte nucleus **(a,b, optical midsection)**. **(c)** Telomere clustering in spermatid nuclei creates a small number of distinct signals. **(e)** Prominent telomere clustering in a Sertoli cell nucleus creates a few large signals. (For details, see ref. **19**.)

## 2.2. Oligonucleotide Probes and Labeling Reagents

1. Telomere probes are obtained from a commercial source as 5'-(TTAGGG)<sub>7</sub> and 5'-(CCCTAA)<sub>7</sub>, deoxyoligonucleotides homologous to the G- and C-rich strand of the vertebrate telomere sequence, respectively (1). Oligonucleotides are preferably labeled by 3'-tailing using terminal transferase (*see Note 1*).
2. Biotin-11-dUTP, 0.4 mM (e.g., Life Technologies, [now Invitrogen], Gaithersburg, MO).
3. dATP, 1 mM (prepared from 100 mM stock, Roche).
4. Cacodylate buffer (5×): 1M potassium cacodylate, 125 mM Tris-HCl, 1.25 mg/mL BSA, pH 6.6. This buffer is usually provided by the enzyme manufacturer, e.g., Boehringer. Cacodylate is a toxic chemical; always handle with care.
5. 25 mM CoCl<sub>2</sub>.
6. Terminal deoxynucleotidyl transferase (Boehringer).
7. TE buffer: 1 mM EDTA, 10 mM Tris-HCl, pH 7.4.
8. Graded ethanol, 70% ethanol/(EthOH).
9. Double-distilled water (ddH<sub>2</sub>O) or MilliQ (Bedford, MA).

## 2.3. Dot Blot Test

1. Nylon membrane (Roche).
2. 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Invitrogen). Dissolve at 50 mg/mL in dimethylformamide; store at -20°C.
3. Nitroblue tetrazolium (NBT, Invitrogen). Prepare stock at 75 mg/mL in 70% dimethylformamide, store at -20°C.
4. AP1 buffer: 0.15M NaCl, 50 mM Tris-HCl, pH 7.5.
5. AP2 buffer: 0.15M NaCl, 50 mM MgCl<sub>2</sub>, 50 mM Tris-HCl, pH 9.5.
6. Substrate solution: mix 4.4 μL NBT, 3.3 μL BCIP, and 1 mL of AP2. Wear gloves when handling dye or substrate solution.
7. Bovine serum albumin (BSA, fraction V; Serva, Heidelberg).
8. Blocking buffer: 1% BSA, 0.1% gelatin in AP1.
9. Streptavidin-alkaline phosphatase (Invitrogen).

## 2.4. Chromosome Preparation

Metaphase chromosomes are prepared from, e.g., cultured blood lymphocytes using standard methanol/acetic acid fixation protocols. Special care for plasma free preparations must be taken.

1. Methanol and glacial acetic acid (Merck, Darmstadt).
2. Fixative; make fresh each time by mixing 3 parts of ice-cold methanol with 1 part of acetic acid.
3. Pasteur pipettes.
4. Plastic centrifuge tubes, 15 mL.
5. Glass slides. Remove impurities by submerging slides for several hours in 80% ethanol in a Coplin jar. Dry with a tissue prior to use.

### **2.5. Cell Suspensions**

When telomere regions are stained and investigated in interphase nuclei, cell suspensions or cells grown on coverslips may be obtained. Cell suspensions are advantageous in that they can be prepared from a particular tissue and nuclei are generally free from neighboring cells and cytoplasm.

1. Acid free 37% formaldehyde (Merck). Formaldehyde is toxic; handle solutions with care.
2. Fixative: 4% formaldehyde, 0.1M sucrose, pH 7.4.
3. RPMI medium (Life Technologies).
4. Ethanol-cleaned glass slides.
5. Fume hood.
6. Agepon (detergent; Agfa, Cologne), 0.1% in deionized water.
7. Small Petri dishes and scalpels.
8. Ice in Styrofoam box.

### **2.6. Pretreatment**

1. Pepsin (3200–4000 U/mg protein; Sigma, St.Louis, MO).
2. Pepsin, stock solution 10 mg/mL deionized H<sub>2</sub>O. Prepare freshly from powder prior to use. Storage at –20°C is not recommended, since enzyme activity will drop with time.
3. Sodium isothiocyanate (NaSCN; Merck). Prepare 1M stock in deionized water. This solution is stable at room temperature for several months when stored in an amber bottle.
4. RNase A (Sigma) stock solution: 10 mg/mL 1×SSC; inactivate DNases by heating for 7 min to 90°C. Store at –20°C.
5. Coplin jars with lids.

## 2.7. FISH

1. 20× SSC: 3M NaCl, 0.3 M Na<sub>3</sub> citrate, pH 7.0. Store at room temperature. Make up fresh 1× SSC and 0.05× SSC from this stock. Discard dilute SSC solutions after use.
2. Formamide, research grade (Merck). A small quantity of deionized formamide is required for the hybridization solution. Small aliquots are prepared by filling the tip of a 1.5-mL microfuge tube with ion exchange resin (20–50 mesh; Biorad, Richmond, VA). Add 1 mL of formamide, mix, and store at –20°C. Note: Formamide is a harmful chemical and should be handled with care (*see Note 2*).
3. 70% formamide, 30% 2× SSC, pH 7.0. The solution can be stored for several weeks in the refrigerator.
4. Carrier DNA from *Escherichia coli* (Sigma), sheared by sonication to 300–1500 bp.
5. Coverslips 22 × 60mm<sup>2</sup> and 13 × 13mm<sup>2</sup>.
6. Coplin jars with covers.
7. Hybridization solution. Consists of 30% formamide, 10% of a 50% (w/v) dextran sulfate (Pharmacia, Uppsala) solution, 10% 20× SSC, 10% 100 mM sodium phosphate, pH 7.0, *E. coli* carrier DNA at a final concentration of 1 µg/µL, DNA probe (labeled G- and C-strand oligonucleotides) at 1 ng/µL final concentration. The amount of slides to be hybridized determines the volume prepared. Use deionized water to adjust volume.
8. Hot plate or heating block.
9. Moist chamber to prevent slides from drying out. Can be, e.g., a lidded plastic box with wet paper and glass rods to raise slides above the moist surface.

## 2.8. Post-Hybridization Washes and Signal Detection

1. Avidin-FITC (e.g., ExtrAvidin-fluorescein; Sigma).
2. Tween-20 (Sigma).
3. BT buffer: 0.15M NaHCO<sub>3</sub>, 0.1% Tween-20, pH 8.3. Make up fresh every day; pH adjustment is not required.
4. BSA (fraction V; Serva) teleostean gelatin (Sigma).
5. Biotinylated goat anti-avidin antiserum (Vector Labs, Burlingame, CA).
6. Water bath, preferably with shaking. Used to adjust the temperature of the solutions in Coplin jars. Always measure temperatures inside Coplin jars.

## 2.9. Reagents for Specimen Counter Staining

1. Propidium iodide (PI; Sigma). Dissolve at 1 mg/mL in sterile water, store at  $-20^{\circ}\text{C}$ .
2. DAPI (4',6-diamidino-2'-phenylindole dihydrochloride) (Sigma), 1 mg/mL in sterile water; store at  $-20^{\circ}\text{C}$ .
3. Antifade solution: Vectashield (Vector Labs) is recommended as mounting medium for fluorescence microscopy, as it reduces fading of fluorochromes efficiently during microscopic analysis (*see ref. 28; see Note 3*).
4. For counterstaining of DNA, dyes are added to the antifade solution in the following concentrations: 0.5  $\mu\text{g/mL}$  DAPI and/or 1  $\mu\text{g/mL}$  PI.

## 3. Methods

This section describes experimental details for probe labeling and detection of telomere repeats by FISH to metaphase chromosomes and interphase nuclei (*see Note 4*).

### 3.1. Probe Labeling

Enzymatic 3' tailing of oligonucleotides has proven an effective method for the generation of FISH probes.

1. Label C- and G-strand oligonucleotides in separate reactions by combining the following:
  - a. 15 pmol oligonucleotide (approx. 160 ng of a 42-mer).
  - b. 1 nmol biotin-11-dUTP and 1.5 nmol dATP (*see Note 5*).
  - c. 10  $\mu\text{L}$  5 $\times$  cacodylate buffer.
  - d. 0.5  $\mu\text{L}$   $\text{CoCl}_2$ .
  - e. 50 U terminal transferase.
  - f. Add ddH<sub>2</sub>O to a final volume of 50  $\mu\text{L}$ .
2. Mix and incubate at  $37^{\circ}\text{C}$  for 3 h – overnight.
3. Set 1  $\mu\text{L}$  of each reaction aside for dot blot test (*see Subheading 3.2.*).
4. Place tubes on ice and combine the C- and G-strand oligonucleotide reactions.
5. Add 20  $\mu\text{g}$  of *E. coli* carrier DNA and 3 vol of ethanol, mix well.
6. Precipitate labeled oligonucleotides along with *E. coli* carrier DNA at  $-20^{\circ}\text{C}$  for 30 min – over night. Additional salt is not required for efficient precipitation.

7. Spin for 30 min at high speed.
8. Discard supernatant and wash pellet once with 70% EthOH.
9. Air-dry pellet by placing open tube for 5–10 min in an incubator or drying block at 65°C.
10. Dissolve pellet in 16  $\mu$ L TE buffer.

### **3.2. Dot Blot Test for Efficacy of Probe Labeling**

The dot blot test is applied to test the efficiency of the labeling reaction (*see* **Note 6**).

1. Spot 6 drops of 9  $\mu$ L 6 $\times$  SSC on a piece of parafilm.
2. Add 1  $\mu$ L of the labeled oligonucleotides (equivalent to 3.2 ng of unlabeled oligomers; *see* **Subheading 3.1.; Step 3**) to the first drop, mix by repeated pipetting in and out of the drop.
3. Transfer 1  $\mu$ L of this drop to the next and mix by repeated pipetting.
4. Repeat **step 3** for the remaining 4 drops.
5. Cut a small piece of nylon membrane.
6. Remove 1  $\mu$ L from the drop with lowest probe concentration and spot it onto the nylon membrane. Repeat this step for drops with increasing probe concentrations.
7. Irradiate the membrane for 30 s on a UV transilluminator.
8. Place the membrane in a small plastic jar, cover with blocking buffer, and incubate for 5 min at 50°C.
9. Pour off blocking buffer and submerge filter in AP1 buffer containing streptavidin–alkaline-phosphatase conjugate (Life Technologies) at 0.5  $\mu$ g/mL. Incubate for 10 min at room temperature with agitation.
10. Discard solution and wash 3 $\times$  for 3min with large volumes of AP1 and 3 min with AP2.
11. Place filter in an appropriately sized plastic bag and add 1 mL of substrate solution.
12. Seal bag and allow the color reaction to proceed in the dark. The dot with the highest oligonucleotide amount (equivalent to 0.32 ng) should become clearly visible within 2–5min, dots 4 and 5 should be visible after 20–30 min; the dot with the lowest amount will remain invisible in most cases.
13. Remove the filter from the bag and stop the reaction by a brief wash in 70% ethanol. Air-dry.

### **3.3. Preparation and Storage of Metaphase Chromosomes**

1. Metaphase chromosomes are obtained from cultured peripheral blood lymphocytes by standard acetic acid/methanol fixation protocols (29). Fixation of the cell pellet with ice-cold fixative should be performed more than 4 times to obtain preparations free of cytoplasm.
2. Drop cell suspension onto slides, tilt, and let excess fixative run down the slide.
3. Using forceps, place the slide onto two glass rods mounted over a water bath at 95°C or a pot of boiling water. Let slides sit in hot steam until the upper side of the slide appears dry (*see Note 7*).
4. Remove preparations and inspect for presence of cytoplasm by phase-contrast microscopy at low power. Cytoplasm is evident as dark rings around metaphase plates and nuclei. Repeat fixation until cytoplasm is absent.
5. Allow dried slides to sit >15 min at room temperature.
6. Seal slides in a plastic container. They may be stored for several months at -20°C.

### **3.4. Preparation of Nuclear Suspension**

1. Obtain fresh or frozen tissue samples.
2. Cut a 4 mm<sup>3</sup> piece from tissue sample and transfer to approx. 1 mL of RPMI medium in a small plastic Petri dish on ice.
3. Mince tissue with scalpels until medium turns turbid.
4. Remove larger tissue fragments with forceps.
5. Transfer 50 µL of the cell suspension to an ethanol-cleaned slide.
6. Mix with 150 µL of ice-cold fixative by tilting slides.
7. Examine suspension by phase-contrast microscopy at low power. Dilute suspension with RPMI if it appears too dense.
8. Allow the solution to dry down in a chemical fume hood.
9. Seal slides in plastic boxes and store at -20°C until ready for use.

### **3.5. Pretreatment and Denaturation of Chromosome Preparations**

1. Place chromosome preparations for 45 min on a heating block or hot plate at 90°C to harden chromosomal chromatin (*see Note 8*).

2. Allow slides to cool to room temperature.
3. Apply 100  $\mu\text{L}$  of RNase solution (100  $\mu\text{g}/\text{mL}$ ), mount large coverslips, and incubate for  $\geq 30$  min at  $37^\circ\text{C}$  (*see Note 9*).
4. Float off coverslips by submerging slides briefly in a Coplin jar with deionized water.
5. Shake off excess fluid and dry, e.g., with an air jet from a rubber blow ball.
6. Cover preparations with 100  $\mu\text{L}$  of 70% formamide/30%  $2\times$  SSC.
7. Mount a  $22 \times 60\text{-mm}^2$  coverslip and incubate for 2 min on a hot plate at  $69^\circ\text{C}$  to denature chromosomal chromatin (*see Note 10*).
8. Pick up slides with forceps, rinse off coverslips and denaturation solution with a jet of cold deionized water from a wash bottle, and air-dry (*see Note 11*).

### **3.6. Pretreatment and Denaturation of Nuclear Suspensions**

This section describes a procedure to achieve an efficient penetration of the probe molecules to their target regions.

1. Submerge preparations (*see Subheading 3.4.*) in a Coplin jar containing 0.1% Agepon and incubate for 5–10min under agitation at room temperature to remove sugar and fixative.
2. Wash briefly in deionized water and drain off excess fluid.
3. Apply 100  $\mu\text{L}$  1M NaSCN and cover with a  $22 \times 60\text{-mm}^2$  coverslip (*see Note 12*).
4. Incubate at  $70^\circ\text{C}$  for 30 min in a humid chamber.
5. Dip wash preparations in  $\text{H}_2\text{O}$  to remove coverslips and NaSCN solution.
6. Add freshly prepared pepsin to prewarmed 0.01 N HCl (final concentration 0.2 mg/mL) in a Coplin jar at  $37^\circ\text{C}$ .
7. Place slides in pepsin solution and incubate for 8 min.
8. Transfer slides to 1% formaldehyde/PBS for 5 min for refixation.
9. Wash 5 min with PBS, 0.1% glycine to saturate free aldehyde groups.
10. Wash preparations in  $1\times$  SSC.
11. Perform RNase digestion as described in **Subheading 3.5., step 3**.
12. Dip wash in  $1\times$  SSC and cover slides with 100  $\mu\text{L}$  70% formamide/ $2\times$  SSC.

13. Mount a 22 × 60-mm<sup>2</sup> coverslip and place slides on a heating block at 85°C (*see Note 13*).
14. Denature for 5 min and remove slides from hot surface with forceps (*see Note 14*).
15. Wash off coverslips and denaturation solution with a jet of ice-cold deionized water (*see Note 11*).
16. Dry slides with an air jet, e.g., from a rubber blow ball.

### 3.7. FISH

1. Denature hybridization solution for 3 min at 93°C.
2. Cool microfuge tube for 3 min in ice water or freezing block. Apply an appropriate volume to a region of the previously denatured slide with the requisite density of metaphase plates or interphase nuclei (3 µL for a 13 × 13-mm<sup>2</sup> coverslip is sufficient).
3. Mount coverslip and seal with rubber cement (*see Note 15*).
4. Place slides for 4–16 h in a moist chamber or incubator at 37°C (*see Note 16*).
5. After hybridization, peel off rubber cement with forceps.
6. Submerge slides in 0.05× SSC at room temperature until coverslips float off.
7. Discard SSC solution and wash preparations 3 × 5 min in 0.05× SSC at 37°C (*see Note 2*).

### 3.8. Signal Detection

The following procedure is a fluorescent detection method that creates green signals at the hybridization site (*see Note 17*).

1. Transfer slides to a Coplin jar containing 0.5% BSA 10.1% gelatin/BT buffer at 37°C and equilibrate for 5 min.
2. Remove slides from Coplin jar and drain excess fluid. Preparations should never dry up during this and subsequent steps.
3. Apply 100 µL BT buffer containing avidin-FITC (2.5 µg/mL); cover with large coverslip.
4. Incubate for 1 h at 37°C in a moist chamber.
5. Shake gently or submerge slides in BT buffer to remove coverslips.
6. Wash slides 3 × for 3 min in BT buffer at 37°C.

7. Apply 100  $\mu$ L BT buffer containing biotinylated goat anti-avidin antibody (2.5  $\mu$ g/ml), cover with coverslip and incubate for 30 min at 37°C in a moist chamber.
8. Wash 3 $\times$  for 3 min in BT buffer at 37°C.
9. Repeat **step 3** and incubate for a final 30 min at 37°C.
10. Repeat **step 8**; remove slides from Coplin jar and drain excess fluid.
11. Apply 18  $\mu$ L antifade solution containing DAPI and PI as DNA-specific counterstains (*see Note 18*) and mount a 22  $\times$  60-mm<sup>2</sup> coverslip.
12. Cover slide with a tissue and apply gentle pressure to remove excess liquid and trapped air bubbles. Inspect under fluorescence microscope (*see Note 19*).

#### 4. Notes

1. By virtue of their small size (<50 bp), telomere deoxyoligonucleotides have proven useful FISH probes to metaphase and interphase telomeres. Commercial ready-to-use telomere FISH kits are available. Short PNA oligonucleotide probes to telomere repeats have yielded superior detection efficiencies (**16**) but are expensive and, because of high formamide requirements, sometimes difficult to combine with other probes.
2. Formamide-containing wash solutions can be replaced effectively with dilute salt solutions (e.g., **19,30,31**), thereby reducing the handling of formamide to the few microliters used in the hybridization solution.
3. Vectashield may be substituted for or diluted by adding an equal volume of antifade solution II. Make up antifade solution II by mixing 245 mg diazabicyclo-222-octane (DABCO, Sigma), 800  $\mu$ L sterile H<sub>2</sub>O, 200  $\mu$ L 1M NaHCO<sub>3</sub> (pH 8.3), and 9 mL high-grade glycerol (86%). These compounds should be added in chronological order, with the NaHCO<sub>3</sub> solution to be added in small quantities, as it may effervesce. DABCO is corrosive; always handle with care.
4. Particular telomeres may be detected by the hybridization of subterminal, chromosome-specific cosmid, YAC-, or microdissection probes (**14,31–33**). dsDNA probes are labeled preferably by nick translation. Genomic probes generally are hybridized *in situ* under suppression conditions, as they frequently contain dispersed repeated sequences (**34–36**). For nick translation and chromosomal *in situ* suppression hybridization, the reader is referred to detailed proto-

cols published elsewhere (37). If oligonucleotide telomere probes are combined with chromosomal *in situ* suppression (CISS) hybridization, a different hybridization scheme must be followed: Telomere FISH is carried out as described. The coverslip is removed after 2 h of hybridization, and the preannealed hybridization solution containing paint and telomere probe is applied to the slide, sealed under a new coverslip, and hybridized for >16 h. It should be noted that the telomere probe concentration in the CISS hybridization solution must be raised to 5–10 ng/ $\mu$ L, as the suppression DNA (usually from the genome from which the paint probe has been generated) will also contain telomere repeat sequences and thereby reduce the hybridization efficiency of the probe at its chromosomal target region(s). In mammalian genomes that harbor telomere repeats at interstitial sites (38), subterminal, chromosome-specific probes are recommended for studying the distribution of particular interphase telomeres.

5. Enzymatic 3' end-labeling of oligonucleotides is compatible with a variety of hapten-modified nucleotides (e.g., digoxigenin or fluorochromes). The sensitivity of oligonucleotide probes may be increased by optimal spacing of haptens within the nucleotide tail, which improves the access of detection agents (fluorochrome-conjugated avidin or antibodies). This is achieved by diluting the hapten-labeled nucleotides with dATP. Note that optimal spacing of the hapten-modified nucleotides depends on the size of the hapten. For example, a molar ratio of approx. 1/6 has been found optimal for digoxigenin-11-dUTP/dATP, whereas the optimal ratio was 1/1.5 in the case of biotin-11-dUTP/dATP (39).
6. If the incorporation of hapten moieties other than biotin are monitored by dot blot, appropriate alkaline phosphatase-conjugated antibodies against the hapten of choice (e.g. digoxigenin) must be used in this test.
7. Well-spread metaphase chromosomes with reproducible performance in FISH are preferably obtained by the steam-dry method (40), as it standardizes preparation conditions (e.g., moisture and drying temperature). Slides are generally stored at  $-20^{\circ}\text{C}$ .
8. Freshly prepared or thawed chromosome preparations are incubated at high temperature to denature and harden chromosomal proteins. This treatment preserves chromosome morphology during the subsequent FISH procedure. Alternatively, chromosome preparations may be fixed for 5 min in PBS/3.7% formaldehyde at room tempera-

ture, washed in PBS/0.1% glycine (v/w), and subjected to pepsin digestion (**Subheading 3.6, step 6**) and FISH.

9. RNase digestion is performed routinely to prevent elevated levels of background fluorescence resulting from unspecific binding of DNA probes to RNA molecules in remaining cytoplasm or in nuclei. This step can be omitted for metaphase chromosome preparations that are free of cytoplasm.
10. Probe and target DNAs can be denatured simultaneously. However, separate denaturation is recommended, especially when the hybridization solution contains dextran sulfate. This polymer creates shearing forces during thermal denaturation that may cause inferior chromosome or chromatin morphology.
11. Ethanol dehydration of chromosome preparations or nuclear suspensions is not required after denaturation. It is sufficient to wash down the coverslip and hybridization solution with a jet of cold deionized water from a wash bottle and briefly air-dry the preparation with an air jet.
12. Successful detection of telomeric regions depends on the access of the DNA probe to its target sequence, particularly in tissues fixed with cross-linking agents like formaldehyde. Access is generally achieved by limited proteolytic treatment and/or treatment with chaotropic reagents such as NaSCN (**41**). Proteinase K and pepsin can be used to remove cytoplasm and nuclear proteins effectively, with pepsin having less detrimental effects on nuclear structure than proteinase K (**30,41**). Pepsin digestion times can be kept at a minimum when, prior to the enzymatic treatment, fixed proteins are dissociated by incubation with chaotropic chemicals such as sodium thiocyanate (**41**). For some applications proteolytic pretreatment can be substituted by incubation with 4× SSC/0.5% Tween-20 (**42**) or dithiothreitol/heparin (**43**), or by repeated denaturation (**27**). FISH to paraffin-embedded tissue section nuclei generally requires proteolytic pretreatment (**19,41**).
13. Preparations fixed with a cross-linking fixative such as formaldehyde require higher denaturation temperature and more time as compared to preparations obtained by standard methanol/acetic acid fixation protocols (**30,41**).
14. During denaturation, coverslips tend to stick to the slide, because of evaporation. This can be prevented by applying several microliters of denaturation solution to the rim of the coverslip.

15. Sealing of coverslips for hybridization is only required for longer hybridization times (overnight or longer). In the absence of rubber cement, coverslips may be sealed with bicycle-tube repair liquid.
16. Although oligonucleotide telomere probes may produce positive signals after only a few hours of hybridization (PNA telomere oligonucleotide probes may create sufficiently intense signals after only 30 min of hybridization), longer hybridization times may result in stronger signals (**44**) and overnight incubations are often convenient.
17. When telomere oligonucleotide probes are combined with other probes, multiple color detection protocols are followed to delineate the chromosomal targets differentially (**14,30**).
18. Mounted slides will maintain their fluorescence up to several months or even years when stored at 4°C or -20°C.
19. Preparations are inspected in an epifluorescence microscope equipped with appropriate filter sets for the visualization of e.g., green, red, and blue fluorescence. Images may be recorded with conventional microphotography or commercial digital image acquisition systems.

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## Telomere Length Distribution

*Digital Image Processing and Statistical Analysis*

**Jean-Patrick Pommier and Laure Sabatier**

### 1 . Introduction

Maintenance of telomere length is a crucial event for cells to survive and proliferate. Telomere length dynamics is an equilibrium between incomplete DNA replication and telomere elongation by telomerase or alternative pathways. Telomere length measurements are usually performed by Southern blotting after enzymatic digestion of genomic DNA, providing terminal restriction fragments. TTAGGG repeat binding factor (TRF) length distribution, thus observed, not only reflects the heterogeneity of telomere length of each chromosome end but also the heterogeneity of subtelomeric regions and the coexistence of different cell populations.

The use of fluorescence *in situ* hybridization (FISH) techniques allows full insight, at the cellular level, of detection of telomeric sequences of individual chromosomes, ruling out the problems raised by cell-to-cell heterogeneity. Strategies based on the use of hybridization of DNA plasmidic probes, oligonucleotidic probes, and large DNA probes generated by polymerase chain reaction (PCR) or DNA ligation are generally employed. The low sensibility

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of these techniques relies on the detection of labeled DNA by several layers of different antibodies. These techniques result in high background and poor quantitative data. Telomeric peptide nucleic acid (PNA) sequences offer high specificity toward pure TTAGGG sequences, avoiding any cross hybridization to degenerate telomeric sequences such as those found in subtelomeric or interstitial regions. Thus, using PNA directly labeled by fluorescein isothiocyanate (FITC) or Cy3 allows detection of a signal directly proportional to the amount of telomere repeats.

Here, a procedure is described for the analysis of the intracellular and intercellular distribution of telomeric FISH signal distribution, focusing on the comparative analysis of individual telomeric signals. A protocol is presented that can be used with free or easy to obtain software and current apparatus in cytogenetic laboratories. All image analysis steps described here are now included in the software (CGA) established from Aphelion Imaging toolkits (ADCIS, Caen, France), which contain an extensive operator library for image processing and image understanding. This procedure has been used successfully to analyze the correlation between telomere length of individual chromosomes and specific chromosome instability.

## **2. Materials**

### **2.1. Software**

1. A software for digital image processing and images acquisition, supporting measurement functions is NIH-Image software (*1*) and is available free from the internet at <http://rsb.info.nih.gov/nih-image>.
2. A software for multivariate statistical analysis is ADE-4 and is available free from the internet at <http://pbil.univ-lyon1.fr/ADE-4F.html> (*2*).
3. The spreadsheet package used is Pro-Fit 5.01, available at <http://www.quansoft.com>.
4. Image software used is Adobe Photoshop 4.0.

## 2.2. Image Acquisition and Computing Material

1. Epifluorescence microscope filter sets optimized for fluorescein isothiocyanate (FITC), cyanine 3 (Cy3) or rhodamine (TRITC), or 4,6-diamidino-2-phenylindole (DAPI) images acquisition and an anti-infrared (IR) filter (*see Note 1*). The image acquisitions were performed with a Leica DMRB microscope which a built-in IR filter.
2. A video or a CCD camera. (*see Note 2*).
3. A GG17 fluorescence standard slide (Karl Zeiss).
4. A printer allowing high-quality paper output such as a phaser printer (Tektroniks).
5. NIH-image, adobe Photoshop 4.0, Pro-Fit 5.01, and ADE-4 were run on a PowerMacintosh 9500/132 (Apple).

## 2.3. Probes

1. FITC or Cy3 coupled through a linker to a (CCCTAA)<sub>3</sub> PNA probe (Perseptive Biosystems, Framingham, MA).
2. *Alu* primer: CGACCTCGAGATCT(C/T)(G/A)GCTCACTGCAA.
3. *Taq* polymerase and Mg<sub>2+</sub> free *Taq* buffer (Boehringer).
4. Genomic DNA (Sigma).
5. Quickspin S400 HR columns for purification of the PCR product (Pharmacia).
6. dUTP-FITC or dUTP-TRITC (Boehringer) if using Cy3-PNA or FITC-PNAprobe.
7. Free dNTP.
8. A thermocycler with an adaptator for slides (DNA Engine, MJ Research) or a heating plate.
9. Nick translation mix for *in situ* probe (Boeringher Mannheim).
10. Hybridization buffer: 50% formamide, 2× SSC, pH 7, 10% dextran sulfate.
11. PNA hybridization mix: 70% formamide, 10mM Tris-HCl, pH 7.2, 1% blocking reagent, 0.3 ng/μm PNA-Cy3 (C<sub>3</sub>TA<sub>2</sub>)<sub>3</sub> probe.
12. Hybridization wash: 70% formamide, 10 mM Tris-HCl, pH 7.2, 0.05M Tris-HCl, 0.15M NaCl, pH7.5, 0.05% Tween-20.

13. Whole chromosome painting probes (WCP; ONCOR or Biosys).
14. Antibody Fab fragments for WCP probe detection: Anti-biotine, Goat + anti-goat/FITC (Vector, Biosys), anti-Digoxigenine/FITC or anti-dig/Mouse+anti-mouse/TRITC (Boehringer).

### 3. Methods

#### 3.1. Chromosome Preparations

1. Add colcemid (4  $\mu\text{g}/\text{mL}$ ) during 1- or 2-hours to actively growing cells to accumulate metaphases.
2. Pellet mitotic cells by centrifuging the culture medium at room temperature for 7 min at 400g.
3. Remove culture medium and resuspend the pellet in 10 mL of warm (37°C) hypotonic solution (10 mL is obtained with 1.25 mL of human serum, 1.5 mL of 75 mM KCl; add water for a total volume equal to 10 mL (*see Note 3*)).
4. Incubate at 37°C for 15 min and then shake tube gently to suspend the cells.
5. Add 0.5 mL of fixative solution (3:1 methanol/glacial acetic acid) and shake gently the tube.
6. Centrifuge at 400g for 7 min at room temperature, discard supernatant medium, and resuspend cells in 8 mL of fixative solution.
7. Repeat **step 6**.
8. Keep the resuspended cells at 4°C until they have spread (*see Note 4*).
9. Wash cells twice with cold fixative solution (**step 6**).
10. After the last centrifugation, resuspend cells in approx 0.5 mL of fixative. Adjust volume as a function of concentration of cells to obtain well-concentrated spread.
11. Spread the cells on wet, ice-cold slides and let the slides dry at room temperature for 24 h.
12. Keep the slides frozen at -20°C until use.

#### 3.2. Alu Probe Preparation

1. Prepare a PCR mixture, in 100  $\mu\text{L}$ , containing 2.5 mM  $\text{MgCl}_2$ , 1 $\times$   $\text{Mg}^{2+}$ -*Taq* Buffer free (Boehringer), 300  $\mu\text{M}$  each dNTP, 2.5 U of *Taq* polymerase (Boehringer), 1  $\mu\text{M}$  *Alu* primer, and 100 ng of placental DNA (Sigma). Perform the PCR as follows: Initial denaturation

(95°C, 3 min), 35 cycles with denaturation (94°C, 1 min), annealing (55°C, 2 min), extension (72°C, 4 min) with 7 min for the last cycle, (*see ref. 3*).

2. Purify the PCR product by DNA precipitation or by centrifugation on a Quickspin S400 HR column (Pharmacia). Evaluate the concentration of the PCR product by spectrophotometry.
3. Label 1 µg of the PCR product by nick translation (nick translation mixture for *in situ* probes, Boehringer) with a direct fluorescent dUTP probe (dUTP-FITC if a Cy3-PNA probe is used). Ratio between dUTP-X and dTTP must be adjusted to 1:3.
4. Precipitate the nick translation product in the presence of 2M ammonium acetate, 2 vol of ethanol, and 50 µg of sonicated salmon sperm DNA, for 2 h at -80°C. Centrifuge at 10,000g for 10 min and discard the supernatant. Air-dry to remove ethanol traces.
5. Resuspend the labeled DNA in hybridization buffer to a final concentration of 5 ng/µL labeled DNA.

### 3.3. Probes In Situ Hybridization

#### 3.3.1. PNA Hybridization

PNA hybridization was performed according to Lansdorp, et al. (4):

1. Allow the slides to dry overnight before use.
2. Immerse the slides in PBS for 5 min at room temperature.
3. Fix slides by immersion 100 mL of 4% formaldehyde diluted in PBS for 2 min at room temperature.
4. Wash in PBS 3 × 5 min for each and add 50–100 µL of pepsin (1 mg/mL in HCl, pH2) and cover the chromosomal spread with a plastic coverslip (*see Note 5*).
5. Incubate 10 min at 37°C, remove the coverslip gently and rinse briefly in PBS.
6. Fix and wash the chromosomal preparation as in **steps 4 and 5**.
7. Dehydrate in ethanol series and air-dry.
8. Lay 10–30µl of PNA hybridization mixture and cover with a plastic coverslip.
9. Perform the telomere hybridization by heating the slide for 3 min at 80°C, followed by 2 h at 25°C.
10. Remove the plastic coverslip and wash twice for 15 min each at room temperature and again 3× for 5 min each at room temperature.

11. Counterstain with 50  $\mu\text{L}$  of DAPI (1  $\text{ng}/\mu\text{L}$  in PBS) for 5 min and cover with a plastic coverslip.
12. Remove the plastic coverslip, rinse briefly in distilled water, add a drop of antifading solution (Vectashield), and mount the slide with a large glass coverslide.
13. The slide can be kept for several days at 4°C and several weeks at -20°C.

### 3.3.2. Unmounting the Slide

After the first round of images acquisition, the slide will need to be unmounted:

1. Remove the oil from the glass coverslide with a paper tissue soaked with ethanol.
2. Shake the slide gently in a 100-mL PBS bath until the glass coverslip can be carefully removed sideways to avoid destruction of the chromosomal preparation.
3. Wash the slide in a second PBS bath to completely remove the antifading solution.
4. Dehydrate the slide in a series of ethanol baths (50%, 70%, and 100%) and air-dry.

### 3.3.3. Alu Probe Hybridization

For subsequent chromosome identification, *Alu*-PCR probe was hybridized to the chromosomal preparation first hybridized with the PNA probe (*see note 6*).

1. Lay 20  $\mu\text{L}$  of denatured *Alu* probe on the slide and cover with a plastic coverslip. The chromosomal preparation does not need to be denatured again.
2. Perform *Alu* probe hybridization for 1 h at 37°C.
3. Wash the slide in 50% formamide, 2 $\times$  SSC, pH 7 (10 min, 37°C) and then in 2 $\times$  SSC (10 min, 37°C).
4. Counterstain as in **Subheading 3.3.1. step 11**.
5. Remount the slide with a drop of antifading solution (Vectashield, Vector) and cover with a glass coverslide.
6. Perform a second round of image acquisitions.

### 3.3.4. Chromosome Painting

1. Unmount the slide as in **Subheading 3.2.2.**
2. Deshybridize the previous probe by leaving the slide in a 100-mL bath of 70% formamide, 2× SSC, at 70°C for 1 min.
3. Wash in PBS.
4. Dehydrate in ascending ethanols baths.
5. Perform hybridization, washing, and detection steps of the WCP probe according to the manufacturer's instructions.

## 3.4. Image Analysis

### 3.4.1. Image Acquisition

Before image acquisition, check that the lamp of the microscope yields a field as homogeneous as possible. It is necessary to perform shading correction (5) with a image of flat-field to correct uneven illumination and a dark current image to detect the presence of hot pixels and noise attributable to CCD reading.

1. Perform the image acquisition with an anti-IR filter, which yields better contrasted images than without filter (*see Note 7*).
2. Record a flat-field image with the GG17 fluorescence standard slide at ×100 magnification.
3. For all fluorochromes, choose the camera settings (exposure time, gain [brighten], offset [darken] for a video camera) to avoid pixel saturation but also to maximize the image contrast. Avoid modifying the settings of the camera between metaphases.
4. Record the coordinates of each metaphase and the exposure conditions (time, gain, offset).
5. For each exposure condition, record a dark current image under the same conditions. To do so, shut off the light and perform the image acquisition.
6. When saving the images, choose image names so that the files can be easily retrieved and submitted to batch processing.

### 3.4.2. Digital Image Processing

#### 3.4.2.1 UNEVEN ILLUMINATION CORRECTION USING NIH-IMAGE

The images used for quantitative analysis should be corrected according to the following equation:

$$I_c = k \times \frac{I_1}{FF_1}$$

$$I_1 = I_{\text{raw}}(t_1) - DC(t_1)$$

$$FF_1 = FF(t_2) - DC(t_2)$$

where :

$I_{\text{raw}}(t_1)$  = the raw image (telomere, exposed for  $t_1$  s,

$I_c$  = the uneven illumination corrected image,

$DC(t_1)$  = the dark current image, exposed for  $t_1$  s,

$FF(t_2)$  = the flat-field image,

$DC(t_2)$  = the dark current image,

$t_1, t_2$  = the exposure times of the telomere and flat-field image  
and, by extension, the settings of the camera, and

$k$  = constant equal to the mean gray level of the  $FF_1$  image.

The correction is done according to the following steps (**Fig. 1**).

1. Load the raw image of the telomere, the flat-field image, and the dark current image.
2. Invert the images.
3. Subtract the  $DC$  image from the  $I_{\text{raw}}$  image with the command *Process/Image Math* (multiplicative factor = 1; additive factor = 0).
4. Subtract the  $DC$  image from the  $FF$  image as in **step 3**.
5. Calculate the mean gray level,  $k$ , of the  $FF_1$  image. Using NIH-Image, this operation can be performed as follows:
  - a. Select all of the gray level—from level 1 to the level 254—of the image with the density slicing tool.
  - b. Select the *mean density* parameter in the *Analyze/Options* menu.
  - c. Perform the measurement with the *Analyze/Measure* command, and determine the value of  $k$  with the *show result* command.

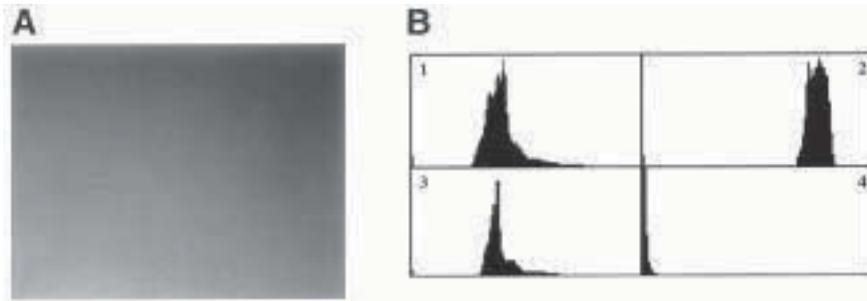


Fig. 1. (A) A flat-field image ( $\times 0.25$ ) with a color LUT, showing imperfections of the filed illumination. (B) Gray level histogramms of  $I$ , Negative raw image of telomeres; 2, negative flat-field image; 3, telomere image corrected for uneven illumination; and 4, corrected telomere image after background subtraction.

6. Divide the  $I_1$  image by the  $FF_1$  image (multiplicative factor =  $k$ ; additive factor = 0). The resulting image,  $I_c$ , is corrected for uneven illumination.

#### 3.4.2.2. CHROMOSOME BANDING ENHANCEMENT

Linear gray level histogram transformation is insufficient to enhance the contrast of the chromosomal banding, as well as for DAPI and *Alu* banding. The following process enhances the banding quality (Fig. 2) and is performed to amplify the mid-range details of the chromosome image.

1. For DAPI or *Alu* images, perform the image modifications on the negative image (Fig. 2 [2]).
2. Stretch the contrast with the *Map* tool (Fig. 2 [3]) so that the gray level of the background is equal to 0 (white) and perform the transformation with the *Apply LUT* (Look Up Table) command.
3. Reduce the size of the image by 20%–50%, (Fig. 2 [4]), depending on the spatial resolution of the image, with the command *Edit/Scale and Rotate* (select the *Create new window* option).
4. Convolve the reduced image by a  $9 \times 9$  pixel Laplace transform kernel (Fig. 2 [5]) with the command *Process/Convolve*, and select the Laplacian  $9 \times 9$  kernel in the kernel folder.

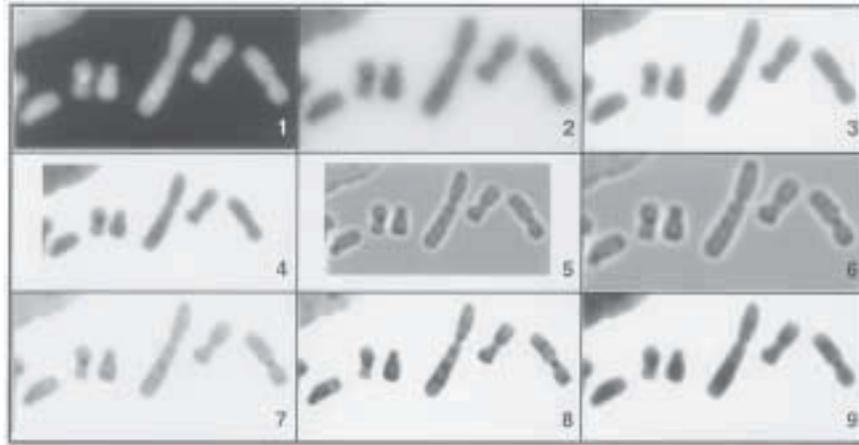


Fig. 2. 1, Raw Dapi image; 2, negative raw Dapi image; 3, previous image with background set to 0 (white); 4, image 3 reduced by 0.8 $\times$ ; 5, image 4 convolved by a  $9 \times 9$  Laplacian kernel; 6, image 5 enlarged by 1.25 $\times$ ; 7, product of the image 3 by the image 6; 8, image 8 linearly contrasted; and 9, image 3 linearly contrasted.

5. Bring the reduced image back to the original image size (**Fig. 2 [6]**) with the command *Edit/Scale and Rotate* (select the *Create new window* option).
6. Multiply (activate the *Real mode* option or choose the multiplicative and additive factor so that the resulting image is not saturated) the resulting image from **step 5** by the resulting image from **step 2** (**Fig. 2 [7]**).
7. Adjust the contrast with the *Map* tool and transform the 32-bit floating point image, if the « *Real mode* » was selected, into an 8-bit image by duplicating the image (**Fig. 2 [8]**). **Figure 2 (9)** shows that a simple linear contrast does not yield DAPI banding as well as that in **Fig. 2 (8)**.
8. For pseudo-color reconstruction, with simultaneous visualization of DAPI and *Alu* banding, it is necessary to register the *Alu* image on the DAPI image. This operation can be done with NIH-Image, but it is easier to register the two images with Photoshop 4.0, as follows:
  - a. Open the DAPI image and convert it into an RGB image; verify that the DAPI image is in the blue channel.
  - b. Invert the DAPI image so that the background becomes black.

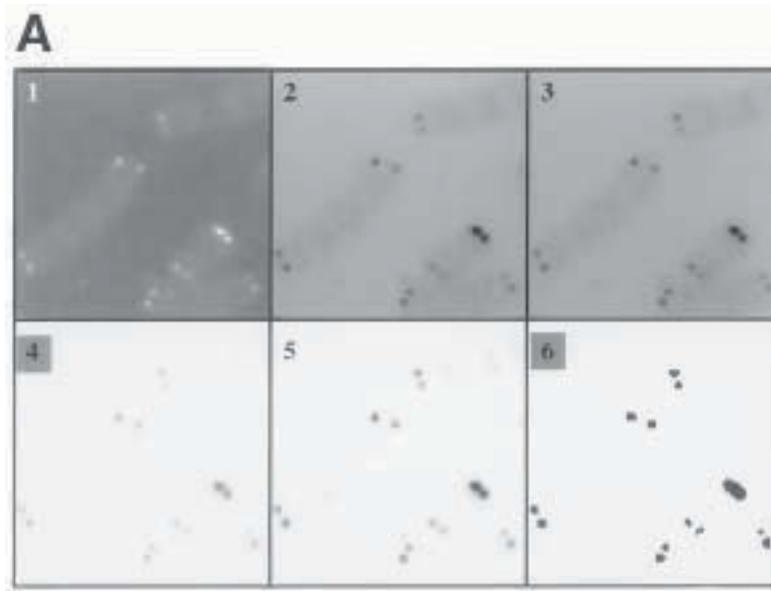


Fig. 3. (A) 1, Raw telomere image; 2, negative raw telomere; 3, telomere image corrected for uneven illumination; 4, corrected telomere image after background subtraction; 5, contrasted image 4; 6, binary image of the telomeres (segmented spots); the images 4 and 6 are used for quantification of the spots intensity.

- c. Invert the green channel so that the background becomes black.
- d. Open the *Alu* image, and copy/paste it into the red channel.
- e. Set the RGB channel tool so that the transformations operate only on the red channel and all colors are visible.
- f. Select a region of interest containing all of the *Alu* hybridization signal, and activate the *Manual transformation* tool.
- g. Perform all Rotation/translation functions necessary to obtain a good image registration.

### 3.4.2.3. TELOMERES IMAGE PROCESSING USING NIH-IMAGE (FIG. 3)

1. Load the telomere image corrected for uneven illumination.
2. Check that the image is a negative so that the objects appear dark on a white background (Fig. 3A).



Fig. 3. (B) A DAPI + telomeres masks image.

3. Set the rolling-ball radius to 5 in the menu (*see Note 8*): *Process/Subtract Background/Set radius*.
4. Subtract the background using the command: *Process/Subtract Background/2D Rolling Ball (Fig. 3A [4])*.
5. Save the background subtracted telomere image « *Telomere-background* ».
6. Duplicate the background subtracted telomere image and enhance the contrast by linear stretching of the gray level histogram of the image, with the *Map* tool (**Fig. 3A [5]**).
7. Perform global thresholding so that all of the telomere spots are detected; the thresholding level must be sufficiently low to detect all of the spots in their entirety but also sufficiently high to avoid noise detection. Transform the image into a binary image with the *Make binary* command (**Fig. 3A [6]**).

8. Save the binary image of the masks of the telomere spots.
9. Because it is easier, in the following telomere measurement step, to visualize simultaneously the detected binary spots and the chromosome, these additional steps can be performed:
  - a. Load a negative DAPI enhanced image.
  - b. Subtract a constant value, equal to 2, to the DAPI image using the command *Process/Arithmetic/Subtract*.
  - c. Add the binary image of the spots to the processed DAPI image. This step can be performed with the logical operator, or in the resulting image, the gray level of the spots must be equal to 255 (with 8-bit images).
  - d. Subtract to the « DAPI+spots » image, a constant value equal to 1.
  - e. Check the *Density slice* option, and select the gray level 254 in LUT. All of the segmented spots will appear red, and, therefore will be selectable for the measurement step; the chromosomes will stay visible (**Fig. 3B**).
  - f. Save the «DAPI+telomere masks» image.

### 3.5. Data Processing

#### 3.5.1. Measuring the Telomeric FISH Signal: Structured Data Collecting

The aim of a structured measurement (see **Note 9**) of the telomere spots is to attribute a given telomere measurement to a given chromosomal arm and to keep this link all through the following data transformations. Because NIH-Image provides the measurement results as a simple list, structuring of the data must be done by the human operator.

1. Choose the parameters in the *Analyze/Options* menu:
  - Area*
  - Mean Density*and check the options:
  - Redirect Sampling*
  - Adjust Areas*
  - Headings*
2. Set the spacial calibration in pixels: *Analyze/Set Calibration/Unit = pixels*.

3. For each metaphase, print the binary image of the spots where the chromosomes are visible (**Fig. 4**).
4. Print a table, «**Table 1**», containing 6 columns and 46 lines (for normal diploid human cells) as follows:

Chromosome Number	Chromosome Name	p Arm Spot left number	p Arm Spot right number	q Arm Spot left number	p Arm Spot right number
-------------------	-----------------	------------------------	-------------------------	------------------------	-------------------------

where Chromosome number = the object number of the chromosome in the metaphase (in normal human cells, 46 objects will be found); Chromosome name = from 1 to X,Y (It might be useful to effect the number 23 to the X chromosome and 24 to the Y chromosome); Spot left/right number = the rank number of a telomere spot when it is measured (for a normal human metaphase in which all of the chromatids are labeled and all of the spots are well separated, this number ranges between 1 and 184).

5. Load the «*Telomere-background*» image.
6. Load the «DAPI+telomere masks» image. No other image must be open.
7. Activate the density slice mode and select the 254 gray level value to highlight the telomeric masks in the «DAPI+telomere masks» image.
8. Choose a chromosome. The first chromosome number is 1.
  - a. Identify the p arm of the chromosome. Write a symbol for p arm on the printed metaphase near it (**Fig. 4A [a]**).
  - b. Write its chromosome number near it on the printed image of the metaphase from **step 3** (**Fig. 4A [b]**).
  - c. Select with the *Wand* tool the left telomere spot of the p arm (**Fig. 4A [c]**).
  - d. Measure the Area and Mean density parameters with the command *Analyze/Measure* (**Fig. 4A [d]**).
  - e. See the result of the measurement with the command *Analyze/Show Result*.
  - f. Write the measurement number corresponding to the left spot in the column «p Arm Spot left number» (**Fig. 4A [e]**).
  - g. Delete from the keyboard the still selected and measured spot (**Fig. 4A [d]**) to avoid measuring the same spot twice.
  - h. Perform the measurement of the right p Arm spot as in **steps 8.c.–g.** (**Fig. 4B [a–c]**).
  - i. Perform the measurements of the spots of the q arm of the chromosome as in **steps 8 c.–i.** (**Fig. 4C**).

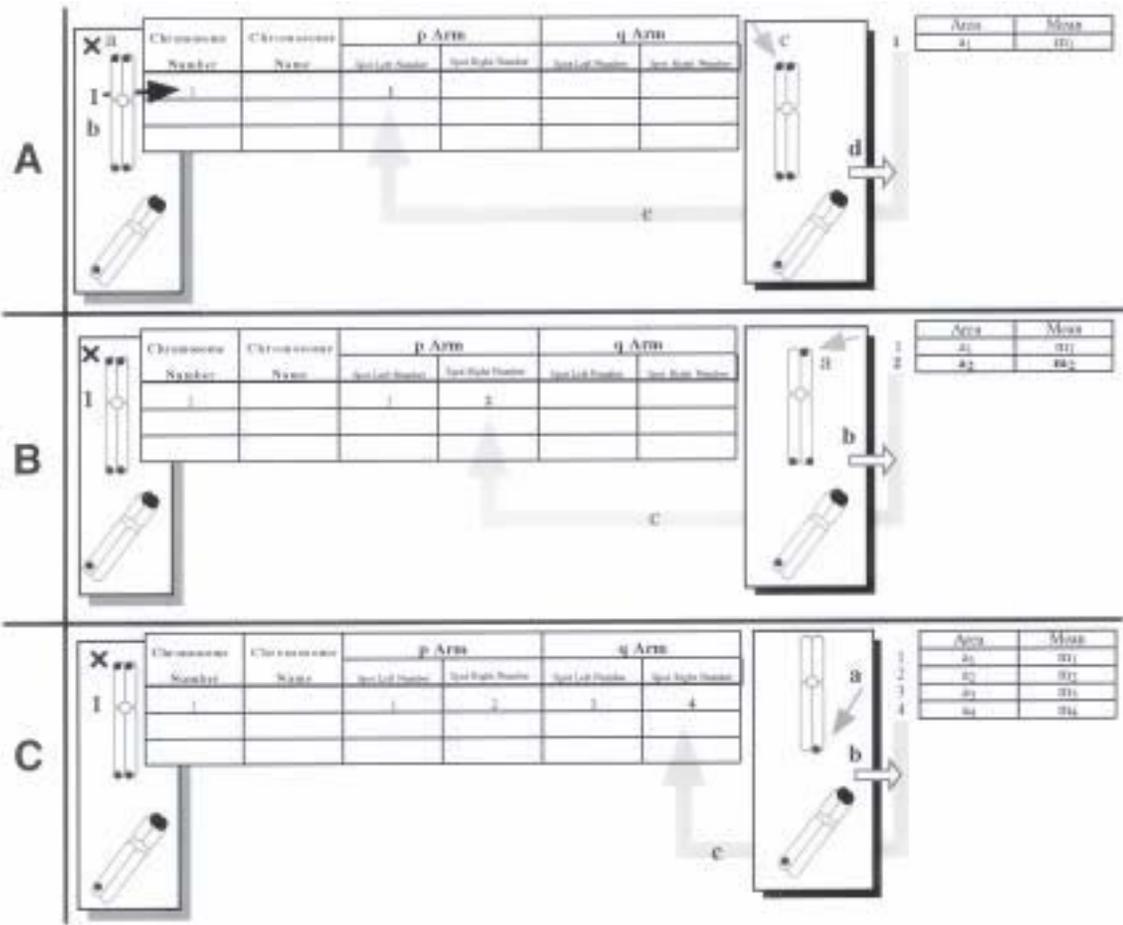
If the two spots of a chromosomal arm are not separated (**Fig. 4D**), do not try to segment them but measure them as a single particule and write a small symbol in «Table 1» indicating that the measurement corresponds to a double spot (ds). Because the parameter is the sum of the intensity of the two telomere spots of each chromosomal arm, the intensity of a ds element is the sum of the intensity of these spots. If only one chromatid owns a detectable spot (**Fig 4E**), write a zero value and indicate ss for single spot at the corresponding location in «Table 1».

9. Choose another chromosome and increase the chromosome number by 1.
10. Write a line from the previous chromosome to the new one (this will be helpfull at the karyotyping step) on the printed metaphase.
11. Perform the spot measurement for the new chromosome as in **step 8 (Fig 4D,E)**
12. Repeat the operation until the last chromosome is processed.
13. Save the results of the measurements with the following file name «Slide-MetaphaseN results-NIH», where «Slide» and «MetaphaseN» are the names of the slide and the number of the metaphase analyzed respectively.
14. Repeat from **step 3** to complete all of the metaphases.

### 3.5.2. Manual Conversion Measurements Results in a New Table: Generation of «Table 2»

1. Run pro Fit 5.01.
2. Load a «Slide MetaphaseN results-NIH» file with text and title options set.
3. Generate a column, called «Intensity», by multiplying the column «Area» by the column «Mean» (**Fig. 5A**).
4. Create a new table, called Table 2, containing 16 columns and 46 lines (**Fig. 5A**). The names of the columns are as follows:

1 – Chromosome Number	9 – p Arm $\sum$ Intensity
2 – Chromosome Name	10 – q Arm $\sum$ Intensity
3 – p Arm Left Spot Intensity	11 – p Arm $\sum$ Standardized Intensity
4 – p Arm Right Spot Intensity	12 – q Arm $\sum$ Standardized Intensity
5 – q Arm Left Spot Intensity	13 – p Arm Flux
6 – q Arm Right Spot Intensity	14 – q Arm Flux
7 – p Arm Spot Type	15 – p Arm Ratio L (L+R)
8 – q Arm Spot Type	16 – q Arm Ratio L (L+R)



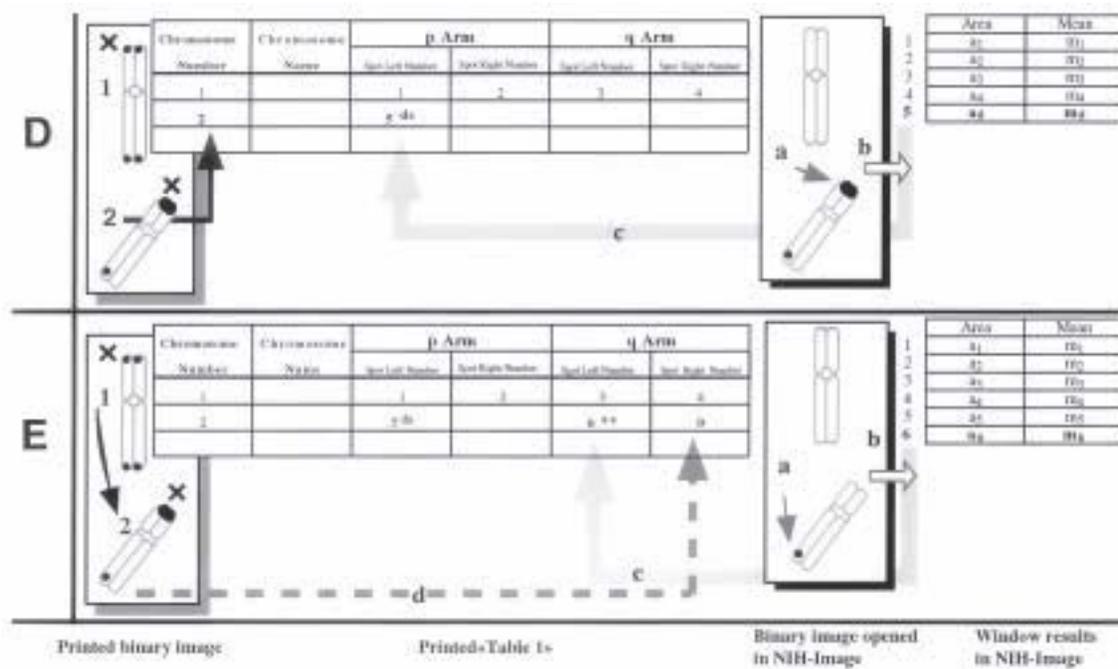


Fig. 4. Structured data measurement (see Subheading 3.5.1).

## A

area	mean	intensity
$a1$	$m1$	$i1 = a1 \times m1$
$a2$	$m2$	$i2 = a2 \times m2$
$a3$	$m3$	$i3 = a3 \times m3$
$a4$	$m4$	$i4 = a4 \times m4$
$a5$	$m5$	$i5 = a5 \times m5$
$a6$	$m6$	$i6 = a6 \times m6$

List of measurements generated by NIH-image after calcul of the intensity values.

Chromosome Number	Chromosome Name	p Arm		q Arm	
		SLN	SRN	SLN	SRN
1		1	2	3	4
2		5ds		6ss	0

Printed Table 1

SLN : Spot Left Number  
SRN : Spot Right Number

1	2	3	4	5	6	7	8	9	10
1	$i1$	$i2$	$i3$	$i4$	1	1	$i1+i2$	$i3+i4$	
2		$i5$		$i6$	0	0	2	$i5$	$i6+0$

Table 2 : See text for the name of the columns.

## B

Detection	Spot Type	Intensity	Sum of the intensities	Ratio
●●	2	$i$	$I=i$	$i / I=1$
● ●	1	$i_L$ $i_R$	$I=i_L+i_R$	$i_L / I$ or $i_R / I$
● ○	0	$i$ 0	$I=i+0$	$i / I=1$
○ ○	0	0 0	$I=0$	Undefined

Type of spot detections per chromosomal arm

- Two spots detected as one particule
- ● One spot detected as one particule
- ○ No spot detected

Fig. 5. (A) Copy/Paste operations from the list of measurements generated by NIH-image, to a «table 2» file using a printed «table 1». (B) Different possible segmentation of the telomere spots and the resulting measurements.

5. With the corresponding «Table 1», copy the intensity value calculated in **step 3** from the «Slide MetaphaseN results-NIH» table to the «Table 2» at the proper location, i.e., in columns 3 or 4 or 5 or 6, as follows. (The *copy/paste* operations are summarized in **Fig. 5A** and process is described in **Note 10**.)
6. In «Table 2», add column 3 to column 4 and put the result in column 9 (**Fig. 5A**).

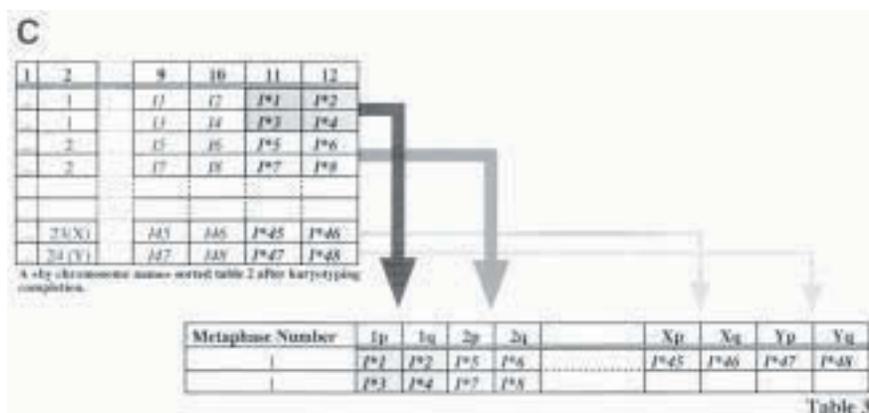


Fig. 5. (C) Copy/Paste operations a « Table 2 » file to a « Table 3 » file.

- In « Table 2 », add column 5 to column 6 and put the result in column 10 (**Fig. 5A**).
- Sort the « Table 2 » by the « p Arm Spot Type », column 7.
- Copy the intensity value, corresponding to ds elements, from column 3 to column 9.
- Sort the « Table 2 » by the « q Arm Spot Type », column 8.
- Copy the intensity value, corresponding to ds elements, from column 5 to column 10.
- Divide the values contained in column 3 by those in column 9 and store the result in column 15.
- Divide the values contained in column 5 by those in column 10 and store the result in column 16.
- Select columns 9 and 10, the columns containing the sum of the intensity of the spots, and calculate the statistical parameters mean and standard deviation (SD), with the command *Calc/Statistics*; select the *Selected cells* option and (at least) the *Basic information* option. Save the calculated parameters.
- Standardize the sum of the intensity of the spots each arm according to the following equation (see **Note 11** and **12**):

$$I^* = \frac{I - \text{mean}}{\sigma},$$

where:

- $I$  = The sum of the telomere spots intensity of each chromosomal arm,  
 $I^*$  = The standardized sum of the telomere spots intensity of each chromosomal arm,

mean = The mean intensity value of the telomere spots intensity of the 92 chromosomal arms,

$\sigma$  = The standard deviation of the telomere spots intensity of the 92 chromosomal arms.

- a. Standardize the sum of the spots intensity of the p arms according to **step 15** with the command *Calc/Data Transformation/Formula*:

$$y = \frac{x - m}{\sigma},$$

where:  $x$  = column 9,

$y$  = column 11

$m$  = Mean sum of the intensity of the spots, and

$s$  = Standard deviation of the sum of the intensity of the spots.

- b. Standardize the sum of the spots intensity of the q arms according to **step 15** with:

$$y = \frac{x - m}{\sigma},$$

where:  $x$ =column 10 and  $y$ =column 12.

16. Divide the intensity values of column 9 by the exposure time of the telomere image and store the result in column 13.
17. Divide the intensity values of column 10 by the exposure time of the telomere image and store the result in column 14.
18. Save the current « Table 2 » with a file name such as «Slide metaphase Table 2», where « slide » and « metaphase » are the name of the slide and the number of the analyzed metaphase respectively.

### 3.5.3. Karyotyping: Completion of « Table 2 » (see **Note 13**)

1. Print on a high-quality output printer the negative DAPI banding enhanced image, its counterpart *Alu* image, or the color DAPI+*Alu* image. If painting were performed, print the corresponding images.
2. With the printed « DAPI+telomere masks » image, where the chromosome numbers were written, choose the first unidentified chromosome and identify it with the images from **step 1**.
3. Write its chromosome name in the column « Chromosome name » of the corresponding « Table 2 ».
4. Complete, if possible, the operation for the 46 chromosomes.

### 3.5.4. Construction of a «Metaphase x Telomeres» Matrix: «Table 3»

The transfer of the telomere standardized intensity from «Table 2» to «Table 3» is summarized in **Fig. 5C**.

1. For a given metaphase, open its corresponding «Table 2» with Pro-Fit 5.0.1.
2. Create a new table, «Table 3», with 2 lines and 49 columns.
3. Name the first column «Metaphase Number».
4. Name the following 48 columns as 1p, 1q, 2p, 2q,..., Xp, Xq, Yp, Yq.
5. Write the number of the metaphase in the first and second lines of the first column.
6. Sort «Table 2» by the chromosome name (e.g. chromosome X is 23, and chromosome Y is 24), so that the parameters of the two chromosomes 1 are located on the 1 and 2 lines of «Table 2», the parameters of the two chromosomes 2 are located on lines 3 and 4, and so on.
7. Copy the Telomere standardized intensity (columns 11 and 12) values from «Table 2» to the proper location in «Table 3», as follows:
  - a. For the first chromosome 1, copy each pair of cells of «Table 2» from line 1, column 11 and line 1, column 12, to the cells of «Table 3» located at line 1, column 1p and line 1, column 1q (*see* the large shaded arrow in **Fig. 4C**).
  - b. For the second chromosome 1, copy each pair of cells of «Table 2» from line 2, column 11 and line 2, column 12, to the cells of «Table 3» located at line 2, column 1p and line 2, column 1q (*see* the large open arrow **Fig. 4C**).
  - c. Complete the copy/paste operation from «Table 2» to «Table 3» for all of the chromosomes (**Fig. 5C**).
8. Open the set of «Table 3» files corresponding to the set of analyzed metaphases and merge the complete set of «Table 3» matrix into a new matrix: «Tables 3 : merged». For  $N$  metaphases, the «Tables 3: merged» matrix will have  $2N$  lines.

### 3.5.5. Plotting Scattering Diagram of the Standardized Telomere Intensity for Each Chromosome

Plotting the scattering diagram of the chromosome 1 as follows:

1. Open the «Tables 3: merged» file with Pro-Fit.
2. With the mouse, select the two first lines of «Table 3» corresponding to the first metaphase.

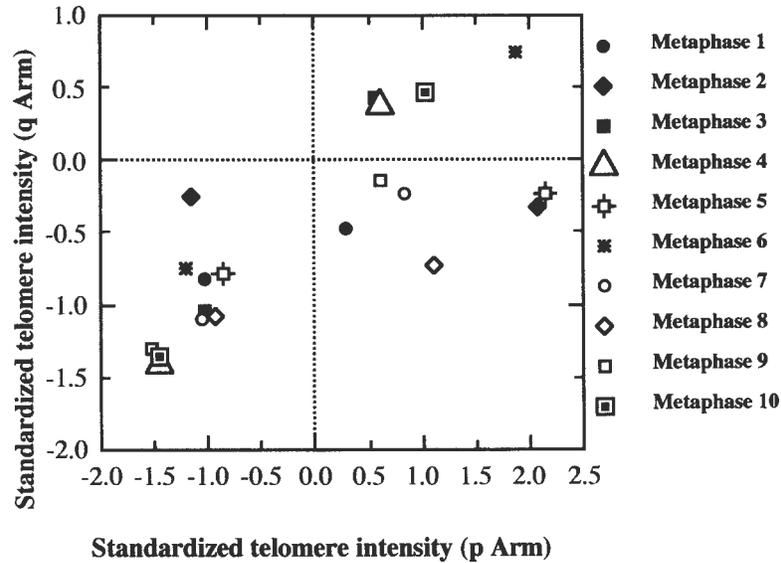


Fig. 6. A scattering diagram of the standardized telomere intensity for the chromosome 1. Each chromosome is represented by a point in the diagram. Each symbol corresponds to a pair of homologue chromosome of a given metaphase.

3. Plot the two points of coordinates (1p, 1q) using the command *Plot data* with the option *Selected rows only*.
4. Select the next two lines of the « Tables 3: merged » matrix, and proceed as in **steps 2** and **3**. Plot all of the pairs of points in the same scattering diagram with a distinct symbol corresponding to each metaphase (**Fig. 6**).
5. Plot the scattering diagrams for all of the chromosomes with one scattering diagram per chromosome.

### 3.5.6. Classification of the Homologue Telomeres by Their Standardized Telomere Intensity

1. For male metaphases, the columns corresponding to the X and Y telomeres will contain  $N$  measurements, although the table contains  $2N$  lines. For columns Xp, Xq, Yp, and Yq and for each pair of lines, duplicate the numerical values so that each pair of lines contains the same values.

2. Remove the first column containing the number of each metaphase. Copy and save the names of the columns (1p,..., Yq) to a text file and rearrange the names of the columns vertically with a text editor.
3. Export the table as a text file.
4. Run ADE-4.
5. Load the text file containing the matrix of data.
6. Transpose the matrix with the command *MatAlg/transpose*. Save the transposed matrix.
7. Open the transposed matrix and compute the matrix of Euclidian distances with the command *Clusters/distances*.
8. Compute the hierarchy file from the matrix of distances with the command *Clusters/Compute hierarchy*. (Choose a second-order aggregation method, such as Ward's method or the "divide" method).
9. Draw a dendrogram to visualize the classification of the telomeres and use the file containing the names of the columns to label each leaf of the dendrogram (**Fig. 7**). Hierarchical classification of the telomeres is performed to provide insights of the intracellular heterogeneity, but the classification is performed on both homologue telomeres (except for chromosomes X and Y if the male metaphases are studied).

#### 4. Notes

1. Filters must be chosen so that the emitted light corresponds to one fluorochrome.
2. Two cameras were used: a video-rate CCD camera (COHU 4912) driven by a Cytovision station (Applied Imaging) and a slow scan cooled CCD camera (Lhesa 1600, KAF 1600 CCD with  $1536 \times 1024$  pixels, Lhesa électronique Cergy-Pontoise, France) driven by Aphelion 2.2 (ADCIS, Caen, France) or with our own custom software for telomere analysis (CytoGenetic Assistant). The raw images exported from a Cytovision station had to be converted into uncompressed 8-bit TIFF for Macintosh, using Photoshop.
3. Using a mix of KCl with human serum produced longer chromosomes preparations than with KCl alone.
4. Cells can be kept for several days at 4°C, but if the chromosome preparation is not optimal, it will be more difficult to obtain well-spread metaphases after 4 d. Cells can also be kept frozen for months.

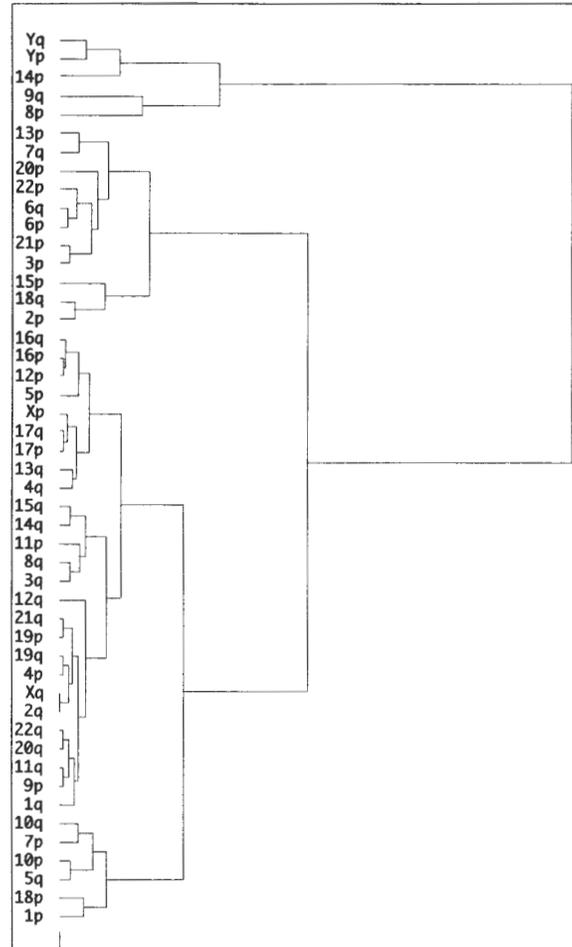


Fig. 7. Hierarchical classification of the homologous telomeres.

5. Pepsine is used at a high concentration (1 mg/mL), and depending on the quality of the chromosomal preparation, it can generate strong degradation in chromosomes morphology. This degradation ranges from destruction of the chromosome banding, to chromosomal swelling, to telomere spots that cannot be viewed simultaneously in the same focus plane. If pepsine treatment results in chromosomal degradation, it is recommended that conditions be altered for chromosome preparation: Lower the temperature of chromosome denaturation, replace the pepsin/HCl solution with a simple HCl (pH 2) solution, or omit the fixation, pepsine, fixation step.

6. The following modification of the telomere hybridization protocol should be followed to avoid a second round of image acquisition with subsequent registration steps between telomeres and *Alu* images required for chromosome recognition. It has not been determined whether the telomere signals are as bright as with the original protocol.
  - a. After PNA probe hybridization, remove the plastic coverslip and dehydrate the slide in ethanol series baths.
  - b. Apply 20  $\mu$ l of denatured *Alu* probe, cover with a new plastic coverslip, and leave the slide at 37°C for 1 h.
  - c. Wash the preparation once in 50% formamide, 2 $\times$  SSC, at 37°C for 5 min and then in 2 $\times$  SSC at 37°C for 5 min.
  - d. Counterstain with DAPI (1 ng/mL) and mount the preparation with an antifading solution (Vectashield, Vector).
7. To open 12- or 16-bit images with NIH-Image, use the command *File/Import*, check the options *Custom* and *16-bit unsigned*, use the command *Set scale* and set «min = 0» and «max = 4095 (12-bits)» or «max = 65535 (16-bits)».
8. Theoretically, different approaches can be envisaged to evaluate the quantity of probes bound to the telomeres. The most complex one consists in using a surface-fitting algorithm for each pair of spots of the metaphase. If the image is considered a surface, where the two first dimensions are spatial and the third dimension corresponds to the gray levels (**Fig. 8A**), the volume under the fitted surface depends on the quantity of the bounded probe (**6**). One-pixel-wide densitometric profiles perpendicular to the chromosome axis, crossing the maximal gray values of the spots, gives a curve whose surface depends also on the bounded probes (**Fig. 8B**). This curve can be submitted to a curve-fitting approach with two gaussian functions (**Fig. 8C**), which give, after revolution around their axis of symmetry, the volume of the pair of spots .
9. Two images are necessary to measure the intensity, or the volume, of the spots: in the first image, the background was subtracted; the second image is a binary image containing the detected spots . The negative raw images of the telomeres were submitted to a local background subtraction, with the *rolling ball* operator, yielding the first image. The size of the *rolling ball* operator must be adjusted (**Fig. 8D**) depending on, the magnification of the objective, the spatial resolution of the CCD (**7**). *Rolling ball* filtering is similar to

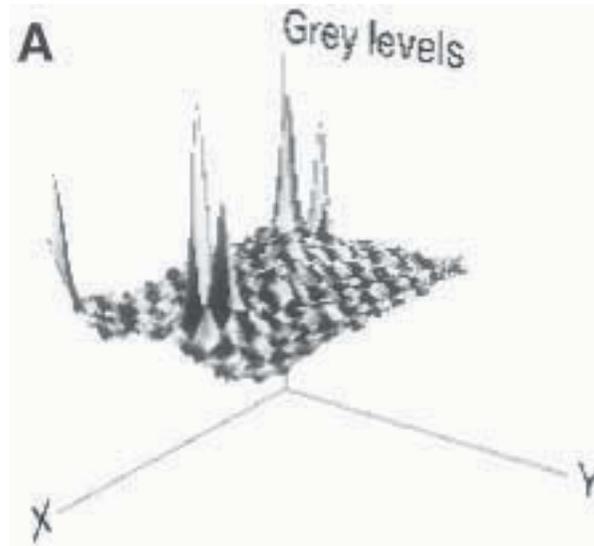


Fig. 8. (A) A telomere image viewed as a surface. The volume of the peaks corresponds to the intensity of a spot (A texture was used for better visualization of the surface).

*TopHat* filtering (8). If *TopHat* filtering is performed, the shape of the structuring element must be chosen as isotrope as possible (i.e., close to the shape of a disk). The following  $7 \times 7$  pixel structuring element are well suited for local background subtraction on telomere images. Depending on the spatial resolution of the image, it can be iterated once to calculate the local background on a  $7 \times 7$  pixel neighborhood and twice for a  $14 \times 14$  neighborhood:



For example with a  $\times 100$  objective, a COHU 4912 video camera (the size of the sensor is  $9\mu\text{m} \times 9\mu\text{m}$  pixels) with a  $0.63\times$  lens in front of the camera sensor, we get a spatial resolution of 7 pixels/ $\mu\text{m}$ .

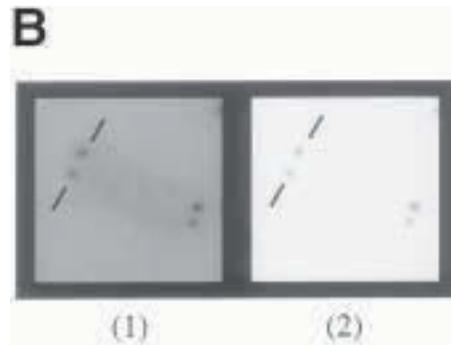


Fig. 8. (B) Details of a corrected telomere image (1), and after background subtraction (2). The lines denote the orientation of densitometric profile.

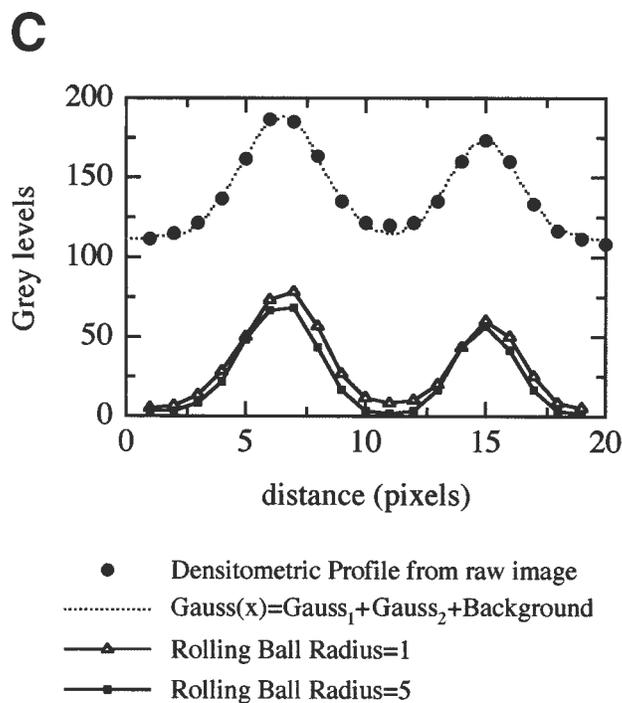


Fig. 8. (C) Densitometric profile from image 1 of (B) (●), fitted two gaussians plus a constant (dashed line). The two others densitometric profiles were extracted from image 1 after background subtraction with a rolling ball of size 1 or 5 (image 2 of [B]).

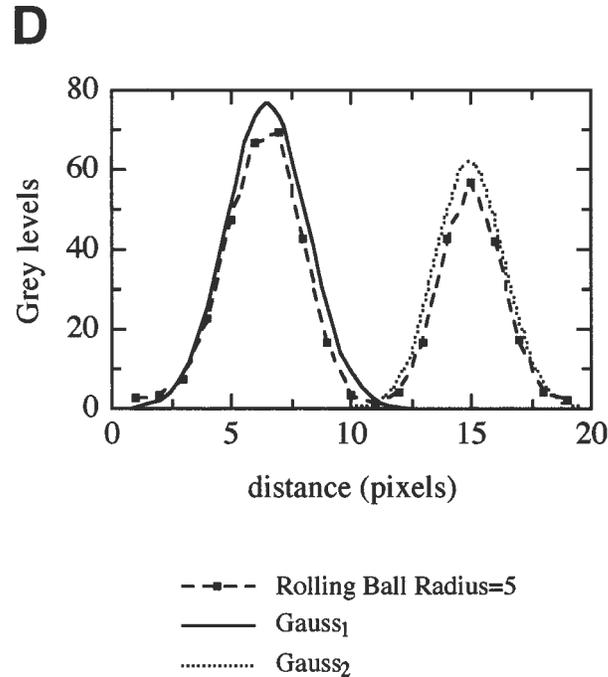


Fig. 8. (D) The fitted function minor the constant is compared to the densitometric profile extracted from image 2 of (B).

10. «Table 2» description:

- a. Read the first (ith) line of the «Table 1».
  - i. Read the chromosome number in the first line (ith line) of the «Chromosome Number» column in «Table 1» and write it in the «Chromosome Number» column of the «Table».
  - ii. Read the «p Arm Left Spot Number» in «Table 1»
    1. If the «p Arm Left Spot Number» correspond to a null value then:
      - a. Write the zero value in the first line (ith line), column 3 of the «Table2».
      - b. Write «0» in the «Table2» at the first line , column 7, see Fig. 5B for the code corresponding to each type of detection.
    2. If the «p Arm Left Spot Number » correspond to a double spot then:
      - a. Read the corresponding line in the «Slide MetaphaseN results-NIH» table.

- b. Copy the corresponding «Intensity» value in the «Slide MetaphaseN results-NIH» table.
      - c. Paste the «Intensity» value in the «Table 2» at the first line (ith line), column 3.
      - d. Leave the cell of the «Table 2» empty at the first line, column 4.
      - e. Write «2» in the «Table2» at the first line , column7.
    3. If the «p Arm Left Spot Number» and «p Arm Right Spot Number» correspond to two single spots then:
      - a. Copy the «intensity» value, in the «Slide MetaphaseN results-NIH» table corresponding to the «p Arm Left Spot Number» from «Table 1».
      - b. Paste the value in the «Table 2» at the first line, column 3.
      - c. Copy the «intensity» value, in the «Slide MetaphaseN results-NIH» table corresponding to the «p Arm Right Spot Number» from «Table 1».
      - d. Paste the value in the «Table 2» at the first line, column 4.
      - e. Write «1» in the «Table2» at the first line , column 7.
    - iii. Read the «q Arm Left Spot Number» in «Table 1» and perform the transfert of the intensity value from the «Slide MetaphaseN results-NIH» table to the «Table 2» as in step 10.ii.
  - b. Read the next (i+1th) line of the «Table 1 » and process the next chromosome according to the steps 10.ii. and 10.iii.
11. The aim of the normalization of the data is to compare the telomere from a given chromosomal arm beyond different metaphases from the same source or from different sources (tissues, donors), relative to the other telomeres of the metaphase.
12. The intensity of a telomere hybridization signal, under linear conditions, depends on the length of the telomere target (the number of bound probes) and on the exposure time:

$$I = \alpha_{\text{optic}} \times \beta_{\text{chr}} \times L_{\text{target}} \times t_{\text{exposure}} ,$$

where

- $I$  = The intensity of the signal in gray levels;  
 $\alpha_{\text{optic}}$  = a constant taking into account the properties of the microscope and of the camera;  
 $\beta_{\text{chr}}$  = A constant taking into account the environment of the DNA target;  
 $L_{\text{target}}$  = The length of the telomere target in kilobases (Kb); and  
 $t_{\text{exposure}}$  = The exposure time of the image in seconds.

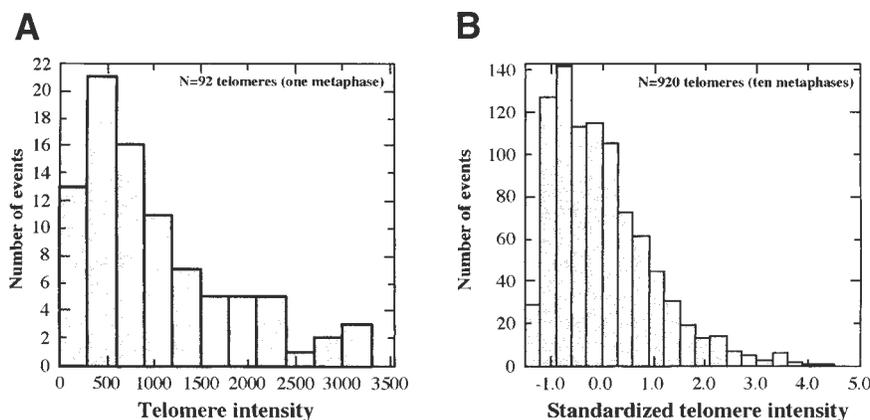


Fig. 9. (A) Histogramm of the telomere intensity (sum of the two spots) values stored in a «Table 2» file. (B) Histogram of the standardized telomere intensity values from 10 metaphases, for intracellular telomere signal heterogeneity evaluation without karyotyping step.

The variable we defined as the flux,  $\phi$ , of the telomere signal,

$$\phi = \frac{I}{t_{\text{exposure}}} = \kappa \times L_{\text{target}} \quad \kappa = \alpha_{\text{optic}} \times \beta_{\text{chr}},$$

allows the performance of external calibration with a target of known telomere length (9,10), to convert the signal (gray levels/s) into kilobase units if the constant  $\kappa$  between the chromosomal preparation and the calibration telomere target remains comparable (11).

13. The aim of the data processing from the raw measurements obtained from NIH-Image to a matrix organized as «Tables 3: merged» is was to have the capability of coding each chromosome by two-component vector so that a chromosome is represented by a point, and a pair of homologue chromosomes by a pair of points in a scattering diagram specific to each of the 24 chromosomes types of the human karyotype. Once the «Table 2» matrix is fulfilled after manual copy-and-paste operations, generation of «Table 3» and the «Table 3: merged» matrix can be automatized using macro commands. The construction of a «Table 3» matrix requires recognition of the chromosomes. Without chromosome recognition, it is still possible for a chosen parameter (intensity, standardized intensity, ratio of intensity of the spots between the chromatids) to compute histograms and to extract global statistical descriptors (mean, mode, SD, skewness (distortion), etc.) of the distribution from «Table 2» (Fig. 9)

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## Analysis of Telomerase RNA Gene Expression by *In Situ* Hybridization

W. Nicol Keith, Joseph Sarvesvaran,  
and Martin Downey

### 1. Introduction

The regulation of telomerase activity is likely to be a complex issue, involving the transcriptional activity of the telomerase RNA component gene, (hTR) and the telomerase catalytic component gene (hTRT), as well as the interaction of telomerase with other telomere-associated proteins (*1–7*), (*see Fig. 1*). The use of telomerase as a diagnostic marker and target for cancer therapy relies on the development of reliable assays and technologies to detect telomeres and telomerase expression (*8–14*), (**Table 1**). Molecular techniques can be roughly broken down into two groups: lysate analysis and *in situ* analysis (*10,15*). With lysate methods, tumor biopsies are homogenized and the spatial relationships between tumor cells are destroyed (Southern blot analysis and polymerase chain reaction [PCR]). This leads to a loss of information on heterogeneity and small subpopulations and presents an averaging of changes. However, quantitation can be simpler and more accurate than *in situ* approaches. In comparison, *in situ* techniques, such as RNA *in situ*

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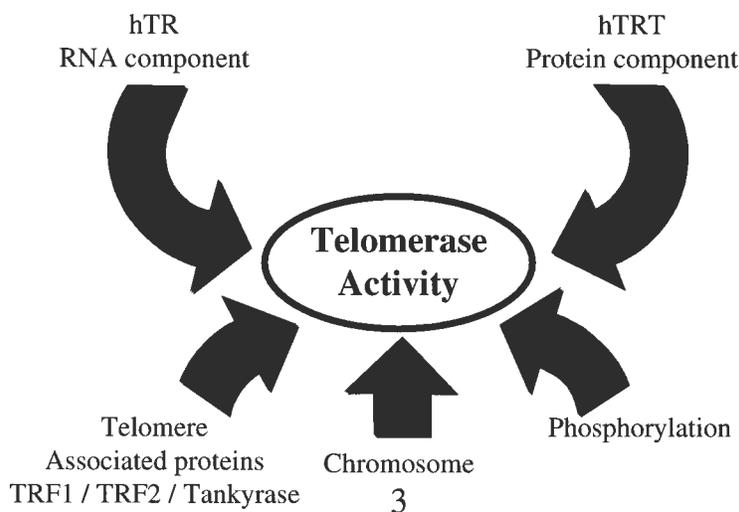


Fig. 1. Regulation of telomerase activity.

hybridization (ISH), allow visualization of gene expression in individual cells within their histological context (10,11,16,17). This is an important issue in examining the role of telomerase in the development of immortal clones of cancer cells from a telomerase-negative normal tissue. Also, for telomerase and telomerase component genes to be useful biomarkers for disease or as a therapeutic targets, differential expression is required between normal and cancerous tissue (11). However, in both normal and cancerous tissue, admixture of cell types may confound interpretation of many assays. The *in situ* approach described here is ideally placed to solve this problem (10,11).

Basic telomerase enzyme activity requires the expression of two genes: the telomerase catalytic component gene (hTRT), and the telomerase RNA component (hTR) (1,6,18). This chapter describes the *in situ* detection of hTR gene expression (10,11); however, GenBank accession numbers for a selection of telomerase and telomerase-associated gene sequences are given in Table 2 (1,3-7), and can be accessed through the internet at <http://www.ncbi.nlm.nih.gov/Web/Genbank/index.html>. Thus, probes to any of the

**Table 1**  
**Methods for Analysis of Telomeres and Telomerase**

Telomerase enzyme activity	TRAP <sup>a</sup> assay
Telomerase component gene expression	Northern blot analysis Nuclease protection assays RT-PCR <sup>b</sup> ISH
Telomere length	Southern blot analysis ISH Flow cytometry

<sup>a</sup> TRAP, Telomeric repeat amplification protocol.

<sup>b</sup> RT-PCR, reverse transcription-PCR.

**Table 2**  
**Sequences of Telomerase and Telomerase-Associated Genes**

Gene	Species	Accession <sup>a</sup> Number	Sequence Length
Telomerase RNA component	human	AF047386	1765
	human	U85256	598
	human	U86046	545
	mouse	AF047387	4044
	mouse	U33831	590
Telomerase catalytic subunit	human	AF015950	4015
	human	AF018167	4027
	mouse	AF073311	3369
	mouse	AF051911	3426
Telomerase associated protein, TP-1	human	U86136	8665
TRF1	human	U40705	2686
	mouse	U65586	1628
TRF2	human	AF002999	2907
	mouse	AF003000	2119
Tankyrase	human	AF082556	4134

<sup>a</sup> GenBank

genes mentioned in **Table 2** can be synthesized and developed in a fashion similar to hTR (*10,11*), (**Fig. 2**).

The principle of ISH is based on the specific binding of a labeled nucleic acid probe to a complementary sequence in a tissue sample, followed by visualization of the probe. This enables both detection and localization of the target sequence. A number of prerequisites for the success of this procedure include the retention of the nucleic acid sequences in the sample and its accessibility to the probe. Specimens suitable for ISH include cells from culture and tissue from samples from whole or biopsied organs. The major steps in RNA ISH are shown in Figure 3.

## **2. Materials**

### **2.1. Linearization of Plasmid DNA**

1. RNA labeling kit (Amersham, RPN 3100).
2. Diethylpyrocarbonate (DEPC) (Sigma).
3. DEPC-treated distilled water (dH<sub>2</sub>O) 1% DEPC; autoclave.
4. Phenol/chloroform/isoamyl alcohol, pH 8.0 (Sigma).
5. 3M Na acetate, pH 8.0.
6. Absolute alcohol, analytical grade.
7. 1% agarose, electrophoresis grade (GIBCO/BRL).

### **2.2. Probe-Labeling**

1. 0.2M Dithiothreitol (DTT) (Sigma).
2. <sup>35</sup>S-labeled UTP (Amersham SJ 603).
3. G50 Sephadex columns (Pharmacia Biotech).
4. Column buffer: 0.3M Na acetate, 1 mM EDTA, 1% SDS; autoclave.
5. Phenol, pH 5.0 (Sigma).
6. Chloroform-isoamyl alcohol (Biogene).
7. 50 mM DTT, aliquoted and stored at -20°C.
8. Scintillation fluid: Eoscint A (National Diagnostics).

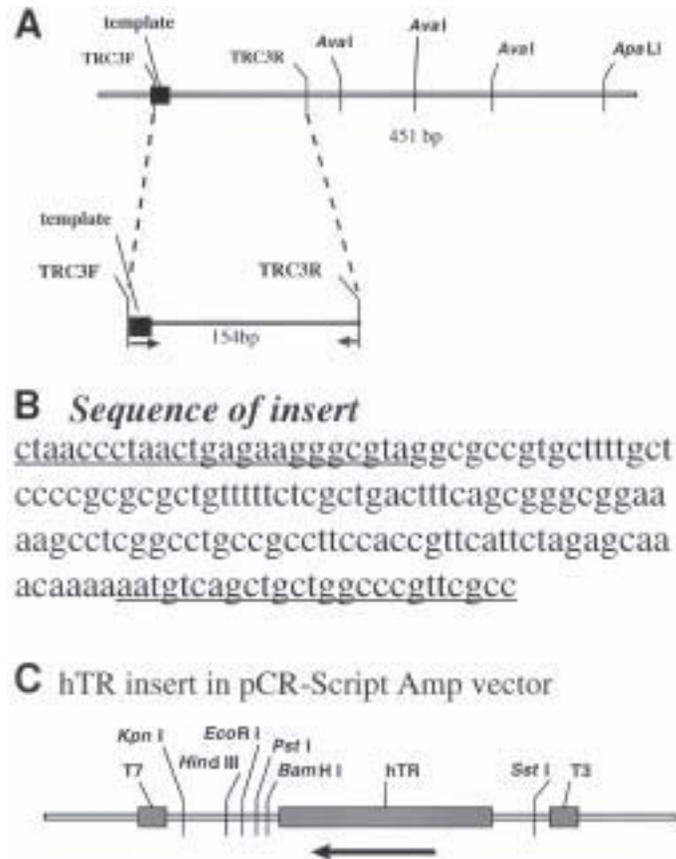


Fig. 2. Development of probe suitable for RNA ISH. (A) Diagrammatic representation of the sequence for the hTR gene. The 154-bp insert used to generate the riboprobe for hTR is amplified with the PCR primers TRC3F (CTAACCCTAACTGAGAAGGGCGTA), and TRC3R (GGC-GAACGGGCCAGCAGCTGACATT). The sequence of this region of the hTR gene is shown in (B), and the sequences corresponding to the PCR primers are underlined. To generate riboprobes, the 154-bp insert was ligated into the Stratagene vector, pCR-Script Amp. This construct is named pCRhTR1, and the region of the vector around the cloning site is shown in (C). The orientation of the insert is shown by an arrow. The antisense probe is synthesized by cutting the plasmid with *Sst*I and using T7 polymerase. Sense probe is synthesized by cutting the plasmid with *Kpn*I and using T3 polymerase. This construct is available from the authors (contact WNK).

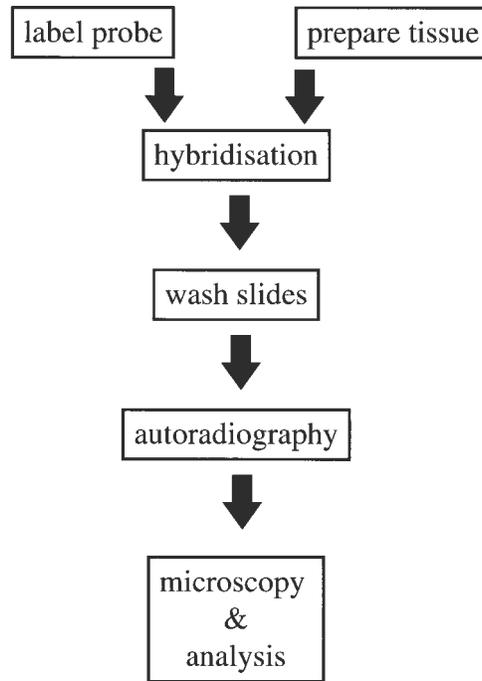


Fig. 3. Overview of RNA ISH.

### 2.3. ISH

1. HistoClear (Fisher).
2. 0.85% NaCl-DEPC: 0.85% NaCl, 1% DEPC; autoclave.
3. 1× PBS: phosphate buffered saline tablets (Unipath), 10 tablets per liter, 1% DEPC, autoclave.
4. 0.5M EDTA, dissolved in DEPC-dH<sub>2</sub>O, pH 7.5.
5. Proteinase K buffer: 1M Tris-HCl, 0.5M EDTA, 1% DEPC, pH 7.5; autoclave.
6. Proteinase K stock solution (Sigma): 20 mg/mL in DEPC-dH<sub>2</sub>O. Aliquot and store at -20°C.
7. Formalin.
8. 0.1M triethanolamine, 1% DEPC, autoclave.
9. Acetic anhydride (Sigma).

10. 1M DTT, aliquoted and stored at  $-20^{\circ}\text{C}$ .
11. 60% hybrid mix: 6 mL of formamide (Fluka), 2 mL of 50% dextran sulfate–DEPC, 1 mL of  $20\times$  SSC, 100  $\mu\text{L}$  of 1M Tris-HCl, 200  $\mu\text{L}$  of  $50\times$  Denhardt's solution, 100  $\mu\text{L}$  of 10% SDS, 400  $\mu\text{L}$  of tRNA (10 mg/mL) (Sigma), 200  $\mu\text{L}$  of salmon sperm DNA (10 mg/mL) (Sigma). Store at  $-20^{\circ}\text{C}$  in 400- $\mu\text{L}$  aliquots.

#### **2.4. Washing Reagents and Solutions**

1.  $20\times$  SSC (GIBCO/BRL); dilute for  $5\times$ ,  $2\times$  and  $0.1\times$  SSC.
2. 50% formamide,  $2\times$  SSC.
3.  $\beta$ -mercaptoethanol (Sigma).
4. RNase buffer: 0.5M NaCl, 1M Tris-HCl, pH 7.5, 0.5M EDTA, pH 7.5.
5. RNase A stock solution (Sigma): 10 mg/mL in DEPC– $\text{dH}_2\text{O}$ , store at  $-20^{\circ}\text{C}$  in 400- $\mu\text{L}$  aliquots.
6. Gelatin: 0.2 g in 200 mL of  $\text{dH}_2\text{O}$ . Microwave for 2 min, filter, and cool.

#### **2.5. Autoradiography**

1. Emulsion for autoradiography (Amersham Hypercoat Emulsion LM-1, RPN 40).
2. Silica gel (Fisher).
3. 20% Phenisol (Ilford).
4. 1% acetic acid (Fisher).
5. 30% sodium thiosulfate (Sigma).
6. Hematoxylin.
7. DEPX mounting solution (BDH).

### **3. Methods**

#### **3.1. Probe Preparation (see Notes 1–6)**

##### **3.1.1. Linearization of Plasmid DNA**

1. Take 10–20mg of DNA and add 10 U of restriction enzyme/ $\mu\text{g}$  of DNA; set up digestion as recommended by suppliers of the enzyme.
2. Leave the reaction at  $37^{\circ}\text{C}$  for 3 h or overnight.

### 3.1.2. Phenol–Chloroform Extraction

1. Add 400  $\mu\text{L}$  of phenol/chloroform/isoamyl alcohol, pH 8.0, and vortex.
2. Spin for 3 min at 15,000  $g$  at room temperature.
3. Keep the supernatant and add 10  $\mu\text{L}$  of 3M Na acetate (pH 8.0).
4. Add 250  $\mu\text{L}$  of 100% ethanol (stored at  $-20^{\circ}\text{C}$ ).
5. Add 1  $\mu\text{L}$  of glycogen to help precipitate the DNA and place on dry ice for 1 h.
6. Spin for 15 min at 15,000  $g$ .
7. Remove supernatant and keep the pellet.
8. Wash pellet with 400  $\mu\text{L}$  of 70% ethanol (stored at  $-20^{\circ}\text{C}$ ).
9. Spin for 10 minutes at 15,000  $g$ .
10. Remove remaining 70% ethanol and air-dry the pellet.
11. Resuspend pellet in 10–20  $\mu\text{L}$  of DEPC- $\text{dH}_2\text{O}$ , depending on the volume of DNA used, for a final concentration of 1  $\mu\text{g}/\mu\text{L}$ .
12. Run 0.5  $\mu\text{L}$  of this suspension on a 1% agarose gel.

## 3.2. RNA Labeling

### 3.2.1. Incorporation of Radioactive Nucleotides

Use Amersham kit no. RPN3100 according to pack insert with reference to the method below (*see Note 8*).

1. Add 4  $\mu\text{L}$  of 5 $\times$  transcription buffer.
2. Add 1  $\mu\text{L}$  of 0.2M DTT and human placental ribonuclease inhibitor (HPR1).
3. Add 0.5  $\mu\text{L}$  of ATP, CTP, and GTP.
4. Add 1  $\mu\text{L}$  of linearized DNA template and 9.5  $\mu\text{L}$  of  $^{35}\text{S}$ -labeled UTP.
5. Add 2  $\mu\text{L}$  of RNA polymerase.
6. Mix and place at  $37^{\circ}\text{C}$  for 1.5 h.

### 3.2.2. DNase Extraction of DNA Template

1. Add 10 U of DNase I.
2. Add 1  $\mu\text{L}$  of RNase inhibitor.
3. Mix and place at  $37^{\circ}\text{C}$  for 10 min.

### 3.2.3. Removal of Unincorporated Nucleotides

1. Equilibrate G50 Sephadex column with 2 mL of column buffer.
2. Add probe to the column.
3. Add 400  $\mu\text{L}$  of column buffer and allow to run through.
4. Add an additional 400  $\mu\text{L}$  of column buffer and collect in an Eppendorf tube.

### 3.2.4. Phenol–Chloroform Extraction

1. Add 400  $\mu\text{L}$  of phenol (pH 5.0), vortex, and spin for 3 min at 15,000  $g$ .
2. Retain the supernatant and to this add 400  $\mu\text{L}$  of chloroform/isoamyl alcohol, vortex, and spin for 3 min at 15,000  $g$ .
3. Retain the supernatant and remove 1  $\mu\text{L}$  for counting the incorporation.
4. Add 2.5 vol of 100% ethanol (stored at  $-20^{\circ}\text{C}$ ).
5. Add yeast tRNA or glycogen to facilitate precipitation of pellet.
6. Place on dry ice for 30 min.
7. Spin for 15 minutes at 15,000  $g$ .
8. Remove alcohol and leave pellet undisturbed.
9. Wash pellet with 70% ethanol and spin at 15,000  $g$  for 10 min.
10. Air-dry pellet and resuspend in 50 mM DTT; calculating the volume as follows:
  - a. Count the incorporation.
  - b. Add 1  $\mu\text{L}$  from **step 5** to 2–3 mL of scintillation fluid.
  - c. Use the formula  $(\text{Actual count} \times 400)/(3 \times 10^5)$ .
  - d. Half the result. This is the volume of 50 mM DTT that the probe should be.
  - e. Resuspended.
11. Count again.

## 3.3. ISH (see Notes 7–9)

### 3.3.1. Pretreatment of Paraffin Sections

1. Dewax with Histoclear; twice, 10 min each.
2. Rehydrate through an ethanol series, 100%, 90%, 70%, 50%, and 30%; for 10 s each.

3. Rinse in 0.85% NaCl and 1× PBS solutions, 5 min each.
4. Proteinase K digest: 400 µL of proteinase K stock solution in 200 mL of proteinase K buffer, 7.5 min.
5. Rinse in 1× PBS for 3 min.
6. Postfix in formalin. Alternatively use 4% paraformaldehyde.
7. Rinse in DEPC-treated dH<sub>2</sub>O for 1 min.
8. Acetylate in 0.1 M triethanolamine with 500 µL of acetic anhydride for 10 min. while stirring under a fume hood).
9. Rinse in 1× PBS and 0.85% NaCl for 5 min each.
10. Dehydrate through the ethanol series; 30%, 50%, 70%, 90% and 100%; 10 seconds each.
11. Air dry.

### 3.3.2. Preparation of Probe and Hybridization (see **Note 6**)

1. For 20 paraffin sections, take 16 µL of 1M DTT, 344 µL of 60% hybrid mix, and 40 µL of probe. Vortex and spin briefly.
2. Denature the probe at 80°C for 3 min. Cool on ice.
3. Apply 20 µL of probe to each section and cover with a glass coverslip.
4. Hybridize at 52°C overnight in a humidified chamber (*see Note 10*).

### 3.3.3. Posthybridization Wash (see **Note 11**)

1. Preheat solutions to required temperature.
2. Wash sections in 5× SSC with 250 µL of β-mercapto ethanol; 30 min at 50°C.
3. Wash in 50% formamide, 2× SSC with 1.4 µL of β-mercaptoethanol for 20 min at 65°C.
4. Wash in RNase buffer, twice 10 min each at 37°C.
5. Wash in RNase buffer with 400 µL of RNase A solution for 30 min at 37°C.
6. Repeat **step 4**, 15 min at 37°C.
7. Repeat **step 3**.
8. Wash in 5× SSC, 0.1× SSC for 15 min each at 50°C.
9. Dehydrate in ethanol series, 50%, 70%, 100% for 1 min each.
10. Air-dry.
11. Dip in gelatin solution, 1 min, and air-dry.

### 3.3.4. Autoradiography (see **Note 12**)

1. Under Kodak Wratten II (or equivalent) safelight conditions, melt emulsion (Amersham LM-1) in dipping vessel immersed in water bath at 46°C.
2. Dip each slide into the emulsion and air-dry.
3. Once dry, place slides in a light-tight box with some silica gel (wrapped in tissue paper), and store at 4°C for 10 d.

### 3.3.5. Development

1. Prepare solutions for development process, with temperature at approx 20°C.
2. Under safelight conditions, develop slides in 20% Phenisol for 2.5 min.
3. Stop development in 1% acetic acid for 30 s.
4. Rinse in dH<sub>2</sub>O for 30 s.
5. Fix in 30% sodium thiosulfate for 5 min.
6. Rinse in several changes of running water for 20 min.
7. Immerse in hematoxylin for 45 s.
8. Rinse in running water for 2 min.
9. Dehydrate in 50%, 70%, and 100% ethanol for 1 min each.
10. Dewax in Histoclear for 10 min.
11. Mount coverslips with DEPX mounting fluid.

### 3.3.6. Analysis

1. Examine sections using light microscopy under light- and dark-field illumination.
2. Score with reference to positive and negative controls.

## 4. Notes

1. Oligonucleotide Probes. These probes, usually 20–30 bp long, can be used. They have the advantage of being fairly easy to generate in large quantities without the need for cloning. Their shorter length enables tissue penetration, and they are fairly stable with no self-hybridization; however, this short length permits fewer labeled

nucleotides to be incorporated per probe and, hence, reduces the sensitivity of the technique. A number of different oligonucleotides targeting different sequences can be used together to increase the signal generated. The hybrids formed by oligonucleotide probes, because of their short length, are also less stable than those formed by RNA probes.

2. **RNA Probes.** Preparation requires use of a DNA template of the target sequence, and generation of sense and antisense RNA probes, with radioactive nucleotides incorporated. Single-stranded RNA probes are ideal if high sensitivity is required; probes of 200–1000 kb have been used, but probes of 150–200 bp are probably optimal, as tissue penetration can become reduced with longer probe size. Limited alkaline hydrolysis can be used to reduce probe size, as required. The RNA–RNA or RNA–DNA hybrids are more stable than their oligonucleotide or DNA counterparts, rendering them the most popular probes.
3. **Probe Label.** Either radioactive or nonradioactive labeling can be used for probes, with autoradiography or immunocytochemistry being the method of detection, respectively. Radioactivity is sensitive with good resolution, with some isotopes offering better resolution but requiring longer exposures, e.g.,  $^{125}\text{I}$ , and others providing results after shorter exposures, but reducing the resolution of the resultant image, because of the wide scatter of the high energy emission particles, e.g.,  $^{32}\text{P}$ .  $^{35}\text{S}$  offers a compromise, with exposure times of 1–2 wk being adequate and providing high-resolution images. Nonradioactive probes offer the advantage of easier working conditions, and digoxigenin-labeled nucleotides can be used to generate the probes.
4. **Checking Probe Size and Specificity.** It is good practice to check the size of a new probe by agarose gel, with nonradioactive nucleotides only. Once the probe is considered appropriate, a Northern blot assay is performed to check its specificity.
5. **Counting the Incorporation of Radionucleotides.** A count of between  $3 \times 10^5$  and  $6 \times 10^5$  is usually found to be satisfactory. In addition, prior to hybridization, the count of the probe should be rechecked. However, although the half-life of  $^{35}\text{S}$  is 90 d, the probe sequence is vulnerable to radiolysis and thus probe performance is optimal only within about 5–7 d of radiolabeling.
6. **Commercial Probes and Control Probes.** Commercially available (Ambion) DNA templates are used for actin and GAPDH to generate

RNA probes for use as positive controls, as they are housekeeping genes and are expressed ubiquitously. Sense probes are commonly used as negative controls and are superior to the omission of a probe as a control. Well characterized tumor samples with a range of RNA expression are used as positive specimens during each run of slides. Commercially available tumor samples of a variety of tissue are also available for the same.

7. Handling RNA. All solutions involved in the preparation of probe and up to the posthybridization wash steps must be free from RNases. Solutions should be treated with DEPC and autoclaved for 4 h at 160°C. This removes the majority of RNases, but by their ubiquitous nature this is not a substitute for care in handling the solutions, glassware and pipets. A dedicated set of pipets for use only with RNA is worthwhile, and regular treatment of the pipets with DEPC-treated water overnight or with a proprietary anti-RNase solution, such as RNaze-Zap (Ambion), may be helpful. All glassware should be autoclaved, by wrapping in aluminium foil prior to use. Plastic Eppendorf tubes may be treated with DEPC-treated water or RNaze-Zap prior to autoclaving.
8. Preparation of Specimen. The objective is to preserve the architecture and morphology of the tissue and retain the RNA products. Rapid processing of the tissue sample either by freezing or fixing in formalin enables RNA to be preserved. Crosslinked fixatives such as 4% paraformaldehyde and 4% formaldehyde are the fixatives of choice for the retention and/or accessibility of cellular RNA. The length of fixation will depend on the specimen size. Longer fixation will result in better tissue morphology, but reduced access to probe may be a consequence. Paraffin wax is the embedding medium of choice. It allows sectioning down to 1 µm in thickness and is removed easily prior to hybridization. As the sections will be processed through a number of solutions during processing, coated slides are recommended. Frozen samples should be frozen down to -70°C and, following cryosectioning, placed on a coated slide and fixed.
9. Prehybridization Tissue Preparation. Preparation of the tissue prior to hybridization helps to increase the access of the probe to the target RNA sequence and reduce non-specific background binding. The specimen is subject to protease treatment to increase the accessibility of the target nucleic acid to the probe, especially if the probe is greater

than 100 base pairs (bp). It is important to postfix the specimen in formaldehyde to prevent disintegration of the tissue. Nonspecific binding to amino groups is reduced by acetylation with acetic anhydride. During tissue preparation, great care must be taken to protect the specimen from RNase. All glassware must be treated to remove any contamination, all solutions treated with DEPC, and gloves worn throughout. Handling of the sections should be kept to a minimum.

10. *In Situ* Hybridization. The hybridization temperature can be critical for some probe/target sequences. Formamide in the hybridization buffer, as a helix destabilizer, reduces the melting point of the hybrids and enables reduction of the hybridization temperature. A low temperature helps preserve tissue architecture; 52°C is optimal. Dextran sulfate in the hybridization buffer, by volume exclusion, increases the concentration of the probe and reduces hybridization times. Although the hybridization reaction is almost complete after 5–6 h, it is convenient to leave the reaction overnight. The sodium ion concentration in the buffer serves to stabilize the hybrids.
11. Post Hybridization Washing. The main objective of posthybridization washing is to remove unbound and nonspecifically bound probe by selection of temperature, salt concentration, and formamide concentration. The use of RNase enables the digestion of single-stranded RNA unbound to target but does not affect the bound RNA–RNA complexes.
12. Autoradiography. Autoradiography enables the detection of the bound probe by radioactivity sensitive emulsion. The emulsion is melted, and a thin layer is used to cover the slide by dipping. The choice of emulsion will depend on the anticipated signal intensity and the method of visualization of tissue, i.e., light or electron microscopy. The slides are dipped into a vessel (Amersham) filled with premelted emulsion (this takes about 10 min). Prior to dipping, a clean slide is used to mix the emulsion slowly and gently. Too rapid agitation will result in the inclusion of air bubbles, which will compromise the quality of the autoradiography. Each slide is dipped for 5 s, removed, and allowed to drain for 5 s, this is then repeated. The back of the slide is wiped gently and the slide placed within a light-tight box on a slide-drying rack for about 60–90 min. Forced drying is not recommended. The temperature of the emulsion at the time of dipping

is critical to the thickness of the emulsion layer. A thin coating of emulsion will increase the resolution of the resultant hybridization. The slides should be dried slowly and under humid conditions to prevent cracking of the emulsion/gelatin and possibly to signal for the formation of silver grains. Replacement of slides in a light-tight box for 2–3 h facilitates slow drying, and when the slides are “tacky” they can be stored in small light-tight containers for the necessary exposure times. Avoid touching the emulsion layer at all times and ensure that slides are not in contact with each other and spaced well apart. After an appropriate length of exposure (about 2–3 wk), the slides can be developed. The exact exposure time may need to be modified, depending on your own results. However, extension of exposure times can increase the sensitivity of the experiment. There may be a resultant loss of resolution and the increase in nonspecific background signal may render the experiment difficult to evaluate. Development of the slides must be carried out in the dark, and on completion, the slides must be rinsed under cold running water. Warm water will melt the emulsion, and the silver deposits will be rinsed off. Although it is safe to switch on the light after the slides have been fixed, rinse the slides in cold water for a few minutes in darkness before switching on the light, as exposure of the fixer to light can cause a yellow/brown discoloration of the slide. Ensure that all reagents used in the development process are at the same temperature and that the working temperature is below 20°C to preserve the gelatin layer. The sections need to be counterstained with hematoxylin to visualize the nuclei. Always ensure that the hematoxylin is freshly filtered.

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## Relative Gene Expression in Normal and Tumor Tissue by Quantitative RT-PCR

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### 1. Introduction

The polymerase chain reaction (PCR) is a very powerful technique for the *in vitro* amplification of nucleic acid sequences (*1*). PCR relies on the principle that oligonucleotide sequences hybridize to a template DNA specifically, given the appropriate conditions. Conditions such as ionic strength and temperature, when optimized, would allow two oligonucleotide primers to hybridize to a DNA template. The primers are complementary to opposite strands of DNA, which allows DNA synthesis by a thermostable DNA polymerase upon hybridization.

PCR consists of three reactions: denaturation, annealing, and extension. The DNA template is denatured at high temperatures to permit annealing of the primers to the template. After annealing, the primers are extended to generate a copy of the template sequence between the two primers. The denaturation step requires a very high temperature (95–98°C), which is why a thermostable DNA polymerase is essential. A continuous cycle of denaturation, annealing

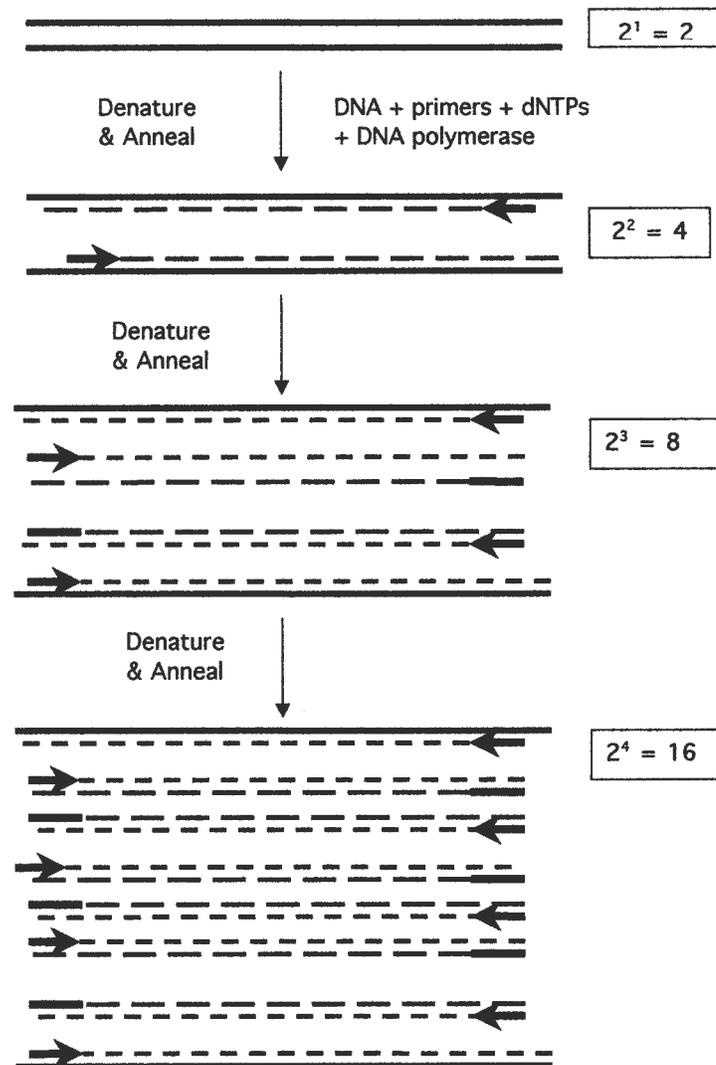


Fig. 1. PCR amplification diagram.

and extension characterizes the PCR. The end result is an exponential amplification of the DNA template (**Fig. 1**) (*see Note 1*).

Quantitative measurement of mRNA using a reverse transcriptase-PCR (RT-PCR) approach has been very useful in gene expression studies. RNA is reverse transcribed to cDNA, and the cDNA is

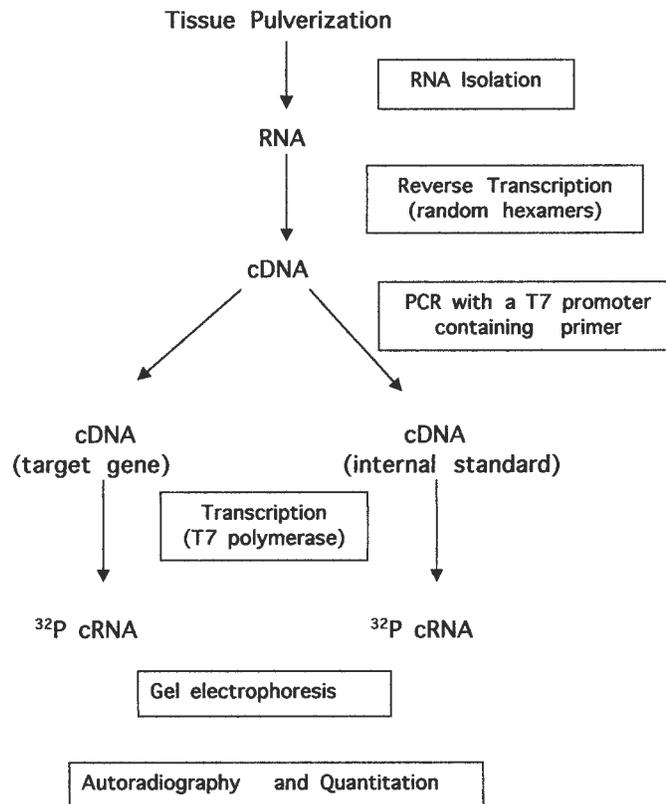


Fig. 2. Schematic diagram of quantitative RT-PCR of relative gene expression.

amplified by PCR. Most of the RT-PCR-based methods determine absolute concentrations of the gene of interest and the internal standard, wherein both genes are coamplified in the same reaction tube. A method to quantitate gene expression of human tumors from small amounts of tissue specimen usually obtained from biopsies was developed in this laboratory (2). In this method, the gene of interest and the internal standard are amplified in separate tubes, which allows for a relative, rather than an absolute, measurement of gene expression.

**Figure 2** shows a schematic diagram of the RT-PCR methodology. Briefly, frozen tumors are homogenized and the mRNA isolated. Reverse transcription of both the gene of interest and the internal stan-

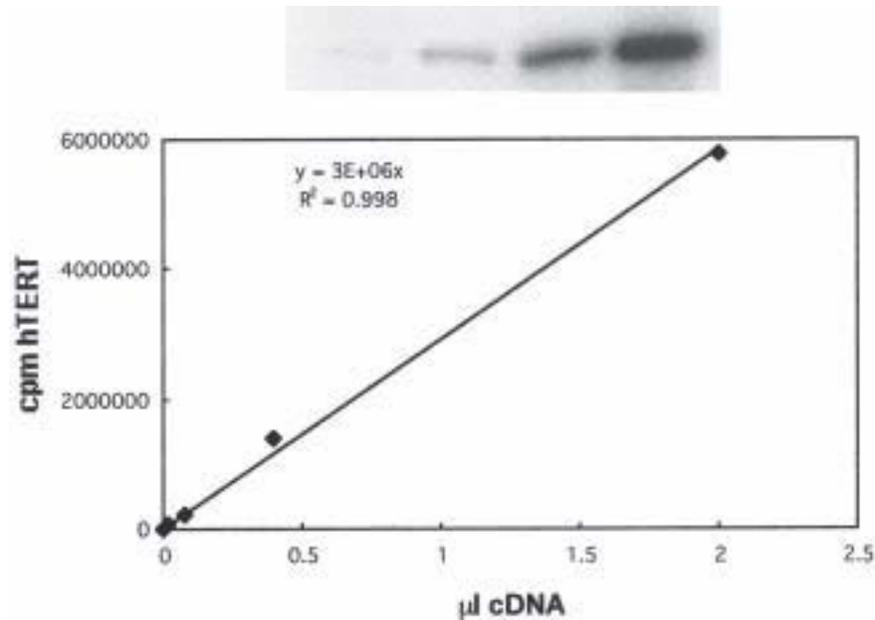


Fig. 3. Representative linear amplification plot for hTERT.

dard is then done in the same tube, which permits an internally controlled reaction. PCR amplification of the gene of interest and the internal standard is then performed in separate tubes. This allows the use of optimized amplification conditions for each cDNA.

Central to the method is the use of a linear range of amplification, wherein the amount of PCR product is linearly proportional to the starting amount of cDNA (**Fig. 3,4**). This is done by serially diluting each sample. The amplified cDNA is then transcribed with T7 RNA polymerase. The 5' end of one primer contains a synthetic T7 promoter sequence and a transcription initiation sequence. It is at this stage that the PCR products are radiolabeled with  $^{32}\text{P}$ . Transcription of PCR products also adds an additional 500-fold amplification of cDNA, thus increasing the sensitivity of the PCR quantitation. The transcribed cRNA can then be analyzed by gel electrophoresis. Quantitation of any gene can be accomplished by finding the linear range of amplification and determining the ratio of its PCR product with that of the internal standard.

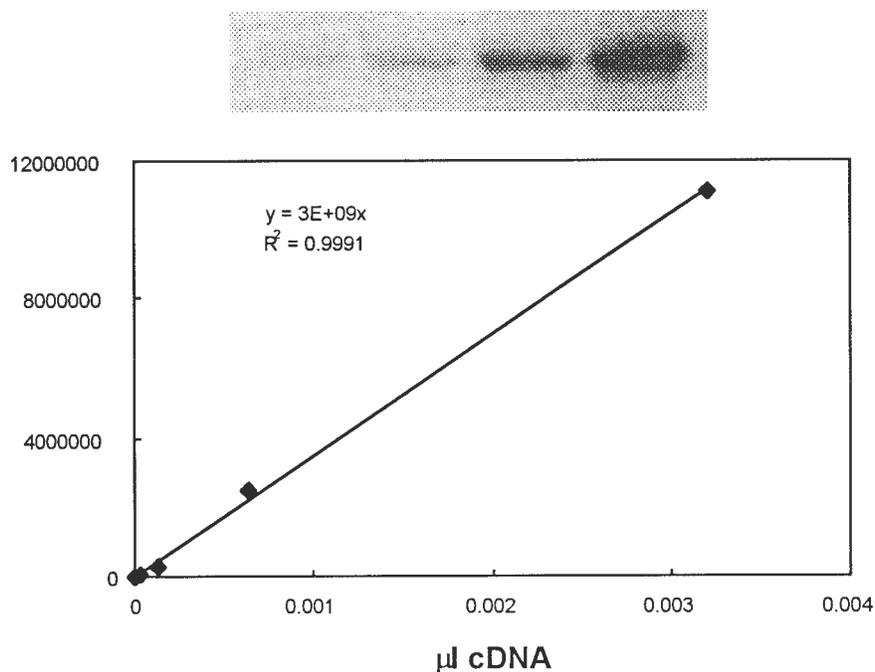


Fig. 4. Representative linear amplification plot for  $\beta$ -actin.

A researcher should be knowledgeable of phosphorimaging technology and its usage before continuing on with data analysis. The equipment for imaging of radiolabeled gels and data acquisition software is provided by Molecular Dynamics, as described in this methodology. Use of other software and technology will also be suitable for data analysis. For more information with regard to use of the Molecular Dynamics phosphorimaging technology, the reader is referred to the company.

## 2. Materials

### 2.1. mRNA Isolation (see Note 2)

1. QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech). Store kit at  $4^{\circ}\text{C}$ .
2. MicroPlex 24 Vacuum Accessory Kit (Pharmacia Biotech).
3. Microplex 24 V-bottom collection trays (Pharmacia Biotech).
4. Steel mortar and pestle.

## **2.2. Reverse Transcription (see Note 3)**

1. 5× M-MLV buffer: 250 mM Tris-HCl, pH 8.3; 375 mM KCl, 15 mM MgCl<sub>2</sub> (Life Technologies).
2. M-MLV reverse transcriptase: 200 U/μL (Life Technologies). Store at –20°C.
3. 100 mM dithiothreitol (DTT), dissolved in water. Store in –20°C (Life Technologies).
4. 10 mM dNTP mix (set of dATP, dGTP, dCTP, dTTP), 1:10 dilution from 100 mM stock (Pharmacia Biotech).
5. Bovine serum albumin: 3 mg/mL in 10mM Tris-HCl, pH 7.5 (Pharmacia Biotech).
6. pd(N)<sub>6</sub> random hexamers: 50 optical density (O.D.), dissolved (Pharmacia) in 550 μL of 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA.
7. RNAGuard ribonuclease inhibitor: 5× 1000 units each (Pharmacia Biotech).

## **2.3. PCR (see Notes 1,4,5)**

1. 10× *Taq* buffer: 500 mM KCl, 100 mM Tris-HCl, pH 8.0, 12.5 mM MgCl<sub>2</sub>.
2. 10 mM dNTP mix.
3. Primers: 12.5 pmol/μL for each 5' and 3' primer (*see Note 6*).
4. Amplitaq *Taq* polymerase (Applied Biosystems). Store at –20°C.

## **2.4. T7 RNA Polymerase Transcription**

1. 10× transcription buffer: 400 mM Tris-HCl, pH 7.5; 120 mM MgCl<sub>2</sub> 0.5 M spermidine; dissolved in water. Store at –20°C (Sigma).
2. 10 mM NTP mixture (set of ATP, GTP, CTP, UTP): 1:10 dilution of 100 mM stock in water (Pharmacia Biotech).
3. 1M DTT, dissolved in water. Store in –20°C.
4. RNAGuard ribonuclease inhibitor: 5× 1000 units each (Pharmacia Biotech).
5. T7 RNA polymerase: 50 U/μL (Epicentre Technologies).
6. (<sup>32</sup>P)UTP Redivue: 3000 Ci/mmol, 10 mCi/mL (Amersham).
7. “Stop” buffer: 24 g of urea, 1.5 mL of EDTA, 320 μL of 4% bromophenol blue, water to final volume of 40 mL.

## **2.5. Gel Electrophoresis**

1. 8M urea.
2. Acrylamide/bisacrylamide (19:1), 40% solution.
3. Gel-loading buffer: 10M urea, 0.02% bromophenol blue.
4. 10× TBE buffer (Life Technologies).
5. 20% ammonium persulfate (APS) with double-distilled water.
6. TEMED.

## **2.6. Data Analysis**

1. Phosphorimager and ImageQuaNT Workstation (Molecular Dynamics).
2. Storage Phosphor Screen Cassette.

## **3. Methods**

### **3.1. mRNA Isolation (see Note 2)**

#### **3.1.1. Initial Steps**

1. Place the mRNA isolation kit at room temperature at least 30 min prior to starting the isolation. The extraction buffer should be placed in a 37°C water bath until all crystalline materials have been dissolved, and then cooled down to room temperature until use.
2. Prepare the oligo(dT)–cellulose by gently swirling the slurry to obtain a uniform suspension. Pipet 1 mL of the oligo(dT)–cellulose into microcentrifuge tubes.
3. Prepare enough 95% ethanol for use (1 mL per sample) and prechill at –20°C.

#### **3.1.2. Extraction of Sample from Tissues (see Note 7)**

1. Place 5–50 mg of tissue in the mortar and pestle. Add liquid nitrogen into the mortar.
2. After the liquid nitrogen has evaporated, immediately pulverize the tissue by striking the pestle with a hammer. Twist the pestle while it is still in the mortar. The tissue must be finely pulverized to obtain a high RNA yield.
3. Remove the pestle and transfer the pulverized tissue to the precooled 50-mL conical tube. Place in dry ice.

4. Add 400  $\mu\text{L}$  of the extraction buffer (600  $\mu\text{L}$  for larger samples). Homogenize well until the tissue is in a uniform suspension.
5. Dilute each sample with 800  $\mu\text{L}$  of elution buffer (1200  $\mu\text{L}$  for larger samples). Homogenize briefly and transfer the homogenate to a sterile 2-mL Eppendorf tube.
6. Place 500  $\mu\text{L}$  of elution buffer per sample at 65°C until needed.

### 3.1.3. Extraction of Sample from Cells ( $1 - 1 \times 10^7$ cells)

1. Add 400  $\mu\text{L}$  of extraction buffer to pelleted cells. Vortex until a homogenous suspension is achieved.
2. Dilute each sample with 800  $\mu\text{L}$  of elution buffer and mix by vortex.
3. Place 500  $\mu\text{L}$  of elution buffer at 65°C until needed.

### 3.1.4. Isolation of mRNA (see **Note 2**)

1. Assemble the Microplex 24 vacuum according to instructions before proceeding with the isolation of mRNA.
2. Centrifuge each sample for 1 min at 14,000g to obtain a cleared homogenate. Tubes containing oligo(dT)–cellulose should also be centrifuged for 1 min.
3. Gently discard the buffer from the oligo(dT)–cellulose by aspiration or by pipeting.
4. Place 1 mL (or 1.5 mL for larger sample volumes) of the cleared homogenate on top of the oligo(dT)–cellulose pellet.
5. Resuspend the oligo(dT)–cellulose by inversion. The oligo(dT)–cellulose may form small clumps. This should be ignored for it will not affect the procedure.
6. Gently mix for 5–10 min by inverting manually or by placing the sample on a rocking device.
7. Place the sample in the microcentrifuge and centrifuge at top speed for 10 s; discard the supernatant by aspiration or pipeting. At this point, the Microplex 24 vacuum assembly should be ready for use.
8. Add 1 mL of high salt buffer to each sample. Resuspend the pellet. Place the tubes in a microcentrifuge and centrifuge for 10 s. Remove the supernatant by aspiration or pipeting.
9. To each tube, add 500  $\mu\text{L}$  of high salt buffer using a pipetor and resuspend the oligo(dT)–cellulose.
10. Transfer the slurry into an empty Microspin column that has been placed in the Microplex 24 vacuum assembly.

11. Engage the vacuum by turning the stopcock just to the point where the liquid begins to evacuate the columns. Leave the stopcock at this position until all the liquid has left the column. Maintain the vacuum for 2–3 s more; then turn the vacuum off.
12. Add 500  $\mu\text{L}$  of high salt buffer to the cellulose pellet. Apply the vacuum as described above. Repeat this step one more time. This washing step can be repeated one to two times, depending on the desired purity of the mRNA.
13. Remove the V-bottom collection tray and replace with a new tray.
14. Add 500  $\mu\text{L}$  of low salt buffer to the cellulose pellet. Engage the vacuum as described above. Maintain the vacuum for 2–3 s after liquid has been evacuated from the column. Turn the stopcock to the “off” position.
15. Repeat the low salt washing step two more times, for a total of three low salt washes.
16. Remove the V-bottom collection tray and replace with a new tray.
17. Add 200  $\mu\text{L}$  of the prewarmed (at 65°C) elution buffer on top of the cellulose pellet. Apply the vacuum as described above. The V-bottom tray now contains the eluted mRNA.
18. Add an additional 200  $\mu\text{L}$  of prewarmed elution buffer to the column and apply the vacuum as described above.
19. Transfer the eluted mRNA to microcentrifuge tubes. The eluted mRNA can now be quantitated, precipitated, or used directly.

### 3.1.5. mRNA Precipitation (see **Note 3**)

1. Add 10  $\mu\text{L}$  of glycogen solution and 40  $\mu\text{L}$  of potassium acetate solution (or 1/10 volume) to the 400- $\mu\text{L}$  sample. Add 1 mL of the prechilled 95% ethanol. Mix the sample by inversion and place it at  $-20^{\circ}\text{C}$  for at least 30 min.
2. Precipitate the mRNA by centrifuging at top speed for 5 min. If the RNA is not to be used immediately, store it at this precipitated state under ethanol at  $-80^{\circ}\text{C}$ .
3. Decant the supernatant and invert the tube over a clean paper towel. Gently tap the tube to assist removal of excess liquid. Air-dry until no liquid droplets remain.
4. Redissolve the precipitated mRNA with 60  $\mu\text{L}$  of 5 mM Tris-HCl, pH 8.0. Thoroughly dissolve the mRNA by careful pipeting. Use filtered tips if possible, to prevent contamination. The mRNA is now ready for reverse transcription, and should be done at once.

**Table 1**  
**Reaction Mixture**

Reagent	Volume ( $\mu\text{L}$ ) per reaction
5 $\times$ M-MLV buffer	20
100 mM DTT	10
10 mM dNTP mixture	10
BSA	2.5
Random hexamers	0.5
RNAguard	2.5
M-MLV reverse transcriptase	5

### 3.2. Reverse Transcription

1. Prepare the reaction mixture for each sample as described in **Table 1**.
2. Place 50  $\mu\text{L}$  of the reaction mixture into 0.2-mL PCR tubes. Transfer 50  $\mu\text{L}$  of mRNA to the tube containing the reaction mixture. Mix by pipeting.
3. Add a drop of mineral oil on the top of each PCR tube. Place the tubes in the thermocycler and start the reaction with the following temperature program: Step 1, 8 min at 26°C; step 2, 45 min at 45°C; step 3, 3 min at 90°C; step 4, 45 min at 42°C; and step 5, 5 min at 95°C. After step 3, the thermocycler should be placed on hold once the temperature goes down to 42°C. Do not take the tubes out of the thermocycler. Add 3  $\mu\text{L}$  of M-MLV reverse transcriptase. Mix thoroughly by pipeting. Proceed to steps 4 and 5 of the reaction. Refer to the Notes section for more information on the reverse transcription temperature program.
5. Store the cDNA at 4°C if the PCR is not to be performed subsequently.

### 3.3. PCR (see Notes 4–6)

1. Prepare master mixtures for each gene of interest. **Table 2** gives an example of a mixture for 50 samples.
2. Prepare and label 8-strip 0.2-mL tubes, one set for serial dilutions of cDNA and another set for the PCR reaction. Prepare enough 10 mM Tris-HCl (pH 8.0).
3. Add the desired volume of 10 mM Tris to the tubes for the serial dilutions (see **Tables 3 and 4**).

**Table 2**  
**PCR Mixtures**

PCR Reagent Mixture (721 $\mu$ L)	Volume ( $\mu$ L)
Water	314
10 $\times$ <i>Taq</i> buffer	194
12.5 mM MgCl <sub>2</sub>	168
10 mM dNTP mixture	45
PCR Primer Mixture	Volume ( $\mu$ L)
PCR reagent mixture	550
Primers (12.5 pmol/ $\mu$ L)	100
(1 $\mu$ L of 5' primer/reaction and 1 $\mu$ L of 3' primer/reaction)(25 pmol/reaction)	

4. Mix the cDNA thoroughly using a pipet or a multichannel pipet. Add the corresponding volume of cDNA serially to achieve either a 10- or 5-fold dilution (see **Tables 3** and **4**). *Note: Prepare the cDNA solution immediately before PCR amplification.*
5. Pipet 10  $\mu$ L of the PCR primer mixture into the PCR tubes. Place 10  $\mu$ L of the diluted cDNA solution into the tube containing the PCR primer mixture. Mix well by pipeting up and down. Add mineral oil (in some cases, addition of mineral oil is not necessary).
6. Place the tubes into the thermocycler and “hot start” the samples with the following preheating program: 5 min at 98°C, 1 min at 70°C, and 1 min. at 60°C.
7. While pre-heating, prepare the *Taq* DNA polymerase enzyme (1:37.5 ratio of enzyme with 1 $\times$  *Taq* buffer ). Add 5  $\mu$ L of the enzyme to the reaction (below the mineral oil, if present).
8. Place caps on the tubes and mix by flicking and vortexing the tubes.
9. Place tubes in the thermocycler and initiate the appropriate PCR program for the gene of interest. An example of a PCR reaction is as follows:  
hTERT: 30 cycles at 96°C for 15 s, 60°C for 30 s, and 72°C for 30 s.  
 $\beta$ -actin: 30 cycles at 96°C for 15 s, 55°C for 30 s, and 72°C for 30 s.
10. When the PCR cycle is finished, store PCR-amplified products at 4°C or at room temperature.

**Table 3**  
**PCR 10-fold Dilution**

Mixture No.	cDNA ( $\mu\text{L}$ )	10 mM Tris-HCl ( $\mu\text{L}$ )	10-fold Dilution, 1 to
1	2	18	1
2	5 from mix 1	45	10
3	5 from mix 2	45	100
4	5 from mix 3	45	1000
5	5 from mix 4	45	10,000
6	5 from mix 5	45	100,000

**Table 4**  
**PCR 5-fold Dilution**

Mixture No.	cDNA ( $\mu\text{L}$ )	10 mM Tris-HCl ( $\mu\text{L}$ )	5-fold Dilution, 1 to
1	2	18	1
2	10 from mix 1	40	5
3	10 from mix 2	40	25
4	10 from mix 3	40	125
5	10 from mix 4	40	625
6	10 from mix 5	40	3125

**Table 5**  
**T7 Transcription Master Mixture**

Reagents	Volume per reaction ( $\mu\text{L}$ )
Distilled water	16.50
10 $\times$ transcription buffer	2.50
10 mM dNTP mix	0.625
1 M DTT	0.25
RNAguard	0.08
0.5 M spermidine	0.10
$^{32}\text{P}$ [UTP]	0.125
T7 RNA polymerase	1.67

**3.4. T7 RNA Polymerase Transcription (see Note 8)**

1. Prepare the T7 transcription master mixture as listed in **Table 5**.
2. Prepare two sets of PCR tubes per sample. Place 20.5  $\mu\text{L}$  of the T7 transcription mixture into one set of PCR tubes.

**Table 6**  
**Denaturing Gel Solution**

Reagents	Amount per gel (40-mL solution)
Urea	19.2 g
10× TBE	4.0 mL
Water	15.5 mL
Acrylamide (40%)	6.0 mL
APS (20%)	200.0 μL
TEMED	9.0 μL

3. Pipet 10 μL of the PCR product into another set of PCR tubes and quick spin. Transfer 3 μL of the PCR product to the tube containing the reaction mixture. This step ensures that no mineral oil is taken up into the reaction.
4. Cap the tubes and gently mix the tubes by flicking and vortexing. Shake down the tube to remove any liquid on the sides of the tube.
5. Incubate for 1 h at 37°C.
6. Add 25 μL of stop reaction buffer and quick spin to mix.

### 3.5. Gel Electrophoresis

1. Prepare the 6% denaturing acrylamide gel solution (*see Table 6*).
2. Prior to adding acrylamide, APS, and TEMED, the gel solution can be heated in a microwave to dissolve the urea. *Note: Do not heat up acrylamide. It is an irritant.* Mix the solution with a magnetic stir bar. Allow the urea to dissolve and the solution to cool down. Acrylamide can be added once the solution has cooled.
3. Assemble the Hoeffer 600 SE vertical gel electrophoresis apparatus according to instructions. Gels can be labeled by inserting cut-up Whatman 3MM papers, with the numbers written in pencil, on the bottom left corner between gel plates.
4. Add the 20% APS and TEMED immediately before pouring the gel. Insert the combs, making sure that no bubbles form below the wells.
5. After the gels have polymerized, rinse the gel plates with water. Take off the combs and rinse the wells with water two to three times. Invert the gel to dry.
6. Assemble the gel plates onto the casting stand. Fill the wells with 1× TBE (1:10 dilution of 10× TBE buffer).

7. Load the samples using gel-loading tips. Fill the electrophoresis chamber with 1× TBE. Electrophorese at 60 mA per gel.
8. After running the gel, transfer onto Whatman 3MM chromatography paper. Label the gels as needed and cover with Saran Wrap™ plastic film. Place the gels on a gel slab dryer and dry for at least 60 min at 80°C.
9. After drying, the gels can be autoradiographed using Kodak XAR film.

### **3.6. Data Analysis**

1. Arrange the dried gels on the storage phosphor screen cassette. Place the phosphor screen on top of the gels and secure it to the cassette. Expose the gels for about 30 min. Remove the phosphor screen and place it on the scanner.
2. After the gels are scanned, the image analysis program will be opened automatically. Enclose each band in a rectangle, making sure that each enclosure does not overlap with the adjacent band. Make the enclosures as small as possible to minimize picking up the background, but large enough to contain the whole band.
3. Generate a report file and export the file in Microsoft Excel format for further analysis. Scanned information can be stored on an optical disk or a Zip disk.
4. Identify the linear range of amplification for every sample by performing a linear regression analysis. The relative expression of a gene is calculated as the ratio between the slope of the linear region of the gene of interest and the slope of the linear region of the internal standard (i.e.,  $\beta$ -actin).

### **4. Notes**

1. One major problem with PCR is contamination. With the ability of PCR to amplify very small amounts of template, contaminants easily can be coamplified and interfere with the reaction, which may result in false-positive data. Always change gloves when necessary to avoid contamination. Be careful not to cross-contaminate reagents and samples. This can be done by having a dedicated working area and a set of pipets and tips exclusively for PCR use.
2. The mRNA isolation kit contains step-by-step instructions. The researcher should refer to the instruction manual for additional infor-

mation. Some reagents, such as the extraction buffer and potassium acetate solution, are irritants and should be handled with care. Some of the oligo(dT)–cellulose resin may be removed during buffer aspiration after centrifugation. This should not be of concern if a small amount of resin is removed. However, if the precipitated resin is disturbed during buffer aspiration, it is necessary to recentrifuge the resin. The 200- $\mu$ L eluate contains approx. 80–90% of recoverable mRNA. The majority of the remaining mRNA is recovered with a second elution step. A second elution step will, however, dilute the mRNA sample. The V-bottom collection trays can be reused for the washing steps. Sterility of the collection trays is not a concern.

3. In preparation for reverse transcription, always make sure that the precipitated mRNA is dissolved completely in 5 mM Tris-HCl. Use of filtered tips is recommended to prevent cross-contamination. Heating the reverse transcription reaction to 90°C denatures the RNA, which increases the efficiency of the reaction, heating the reaction to 95°C inactivates the nuclease activity of the M-MLV reverse transcriptase. Addition of more reverse transcriptase increases the yield of cDNA.
4. When performing PCR, handling of numerous samples can be time-consuming. The use of a multichannel automatic pipet (Rainin Instruments) and the use of 8-strip PCR tubes (Robbins Scientific) has proved to be helpful. Mineral oil also has been used on top of the reaction mix. This may not be necessary, depending on the thermocycler being used. This step should be established by running duplicate tubes with and without mineral oil to assess the problem.
5. The PCR assay is central to the whole quantitation procedure. In this step serial dilutions are made and a linear range of amplification is expected. Each gene of interest should have an optimized PCR cycling parameter to ensure specific amplification of the gene of interest, minimal nonspecific PCR products, and the longest linear range. This can be done by running different annealing temperatures to determine which temperature works best. The number of cycles is also important. Too many cycles may result in a plateau effect on the amplification of PCR products. Although 30 cycles is usually optimal, some genes may require more cycles.
6. Primers are usually designed to span a region of about 100–200 bases, and if possible, to span adjacent exons to minimize genomic DNA amplifications. Each 5' primer also has the sequence TAA-

TACGACTCACTATAGGGAGA attached to its 5' end. The first 17 bases are the synthetic T7 promoter sequence, and the latter 6 bases are the transcription initiation sequence.

7. Mortars and pestles should be kept cold and in dry ice 1 h before the isolation procedure. Also precool one 50-mL disposable polypropylene conical tube in dry ice for each sample. Tissue samples should be kept frozen at all times, as the mRNA should be retained in the column until the final elution step.
8. DTT and spermidine are hygroscopic reagents, especially spermidine. It is best to prepare the reagents in aliquots. Make sure to thaw the DTT and spermidine solution completely before opening the tube for use. It is imperative that special handling should be considered for radioactive material, both in transcription and in gel electrophoresis. For multiple gels, a multigel caster system (Hoeffer Scientific) that can assemble up to 12 gels can be used. Use of this system provides ease in handling and eliminates the possibility of leakage.

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## **Quantitative Detection of Telomerase Components by Real-Time, Online RT-PCR Analysis with the LightCycler**

**Thomas Emrich, Sheng-Yung Chang,  
Gerlinde Karl, Birgit Panzinger, and Chris Santini**

### **1. Introduction**

Telomeres are specialized DNA/protein structures, located at the ends of eukaryotic chromosomes, that consist of small, tandemly repeated DNA sequences. They have an essential role in the stable maintenance of the eukaryotic chromosome by preventing nucleolytic degradation, end-to-end fusion, irregular recombination, and other events that are normally lethal to a cell (**1**). In most normal tissues telomeres progressively shorten with each replication cycle as a result of the end-replication problem (**2**). Because germ-line cells, stem cells, and especially tumor cells exhibit a prolonged or even infinite life span, it was proposed that these cells must possess a particular mechanism for maintaining telomere length (**3**).

The maintenance of stable telomere length is associated with the activation of telomerase. Telomerase is a ribonucleoprotein that compensates for the loss of telomeric DNA by adding repeat sequences to the chromosome ends using its intrinsic RNA compo-

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ment as a template for DNA synthesis (4). Using the highly sensitive PCR-based telomeric repeat amplification protocol (TRAP) method (5), telomerase activity has been detected in most neoplastic lesions and appears to be necessary for the sustained proliferation of most advanced cancers (5,6). Therefore, the presence of telomerase activity in tumors may be a useful marker for diagnosis and prognosis of cancer (6).

The measurement of telomerase activity by the TRAP assay is limited by several issues with respect to (1) the sample material, including the requirement of enzymatically active telomerase, variances in lysis efficiency, instability of activity during storage (7); and (2) the assay itself, including unspecific amplification products (8), inhibition of the assay by PCR inhibitors from the sample (9), and limitations in quantitation of the assay. These technical limitations could be overcome by indirect measurement of telomerase via its corresponding gene products.

The genes encoding the telomerase RNA subunit (hTR) and the human telomerase catalytic protein subunit (hTERT) have been cloned from a variety of species, including humans (10–12). Both subunits are essential for restoring telomerase activity *in vitro*, and introduction of these genes into normal human cells can extend the life span of these otherwise mortal cells (13,14). Furthermore, correlation studies using a qualitative reverse transcriptase–polymerase chain reaction (RT-PCR) assay have shown a significant relationship between telomerase activity and hTERT expression, e.g., in urothelial cancers (15). Similar correlations have also been demonstrated for hTR expression in tumors (16), although in some instances hTR may also be expressed at lower levels or even independent of enzyme activity, indicating the need for a quantitative approach for assessing hTR (17).

This chapter describes a convenient, nonradioactive RT-PCR method for the detection of the hTR and hTERT. Using a closed-tube, rapid PCR amplification and real-time fluorescence detection system (LightCycler; 18,19), hTR and hTERT levels can be specifically and quantitatively detected with 10- to 100-copy sensitivity in less than 45 min.

As shown in **Figure 1**, the relative fluorescence increases in reactions containing serially diluted *in vitro* RNA transcripts coding for hTR or hTERT. Both assays show a linear relationship between the cycle number and the logarithm of the copy number per reaction over a range of at least 5 magnitudes, covering  $10^6$  to  $10^2$  hTR or hTERT copies per reaction. Using these hTR- and hTERT-specific *in vitro* RNA transcripts as external standards, hTR and hTERT RNA levels of different cell lines were analyzed, and it was shown that elevated expression levels of hTERT and hTR transcripts are found in telomerase-positive HeLa and 293 cells in contrast to telomerase-negative HuVec (human umbilical vein endothelial) cells (**Fig. 1**).

As hTERT primers were specifically designed to span an exon-intron boundary, the signals observed were highly specific for hTERT RNA transcripts. This specificity was also indicated by the absence of any amplification products in the reverse transcriptase-negative (–RT) control reactions. In contrast, because of the lack of introns in the hTR gene, hTR-specific primers that differentiate between amplification from RNA transcripts and genomic DNA contaminations in the sample cannot be designed (**10**). In this case, the amount of hTR amplification observed in the (–RT) reactions that are derived from contaminating genomic DNA can be calculated easily by the method described below (*see Fig. 1*). Furthermore, using the RNA preparation methods described below, typical DNA contaminations of less than 0.1% were observed, indicated by the presence of signals in very late cycles of the PCR process (**Fig. 1**).

In addition, the real-time PCR technology described here can be adapted easily to the quantitation of housekeeping gene transcripts in the same RNA preparation. As the methods are extremely sensitive, in contrast to most conventional methods, LightCycler PCR is extremely advantageous when only limiting amounts of sample material, e.g., mRNA preparations from biopsies, are available.

As the on-line PCR method presented here allows rapid amplification and on-line detection of the amplification products in only 45 min, it is especially useful for rapid screening of large numbers of clinical samples and is thus ideal for elucidating the role of telomerase in the diagnosis and prognosis of cancer.

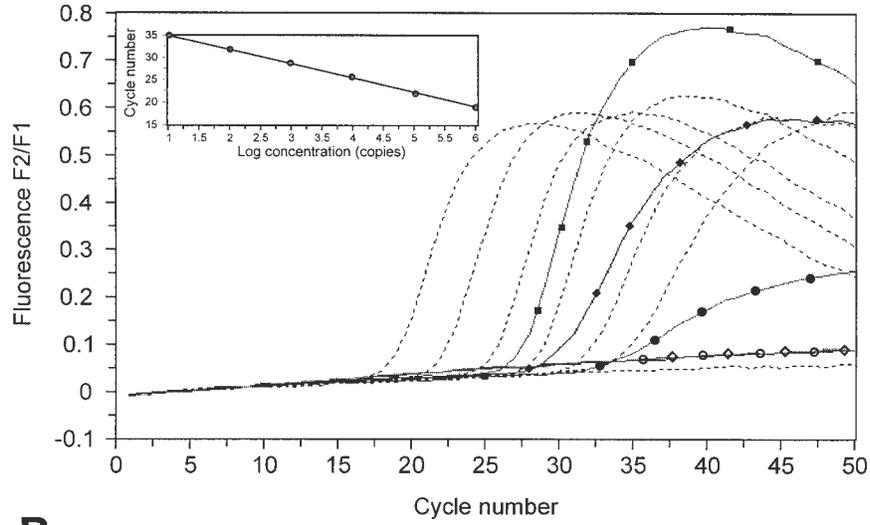
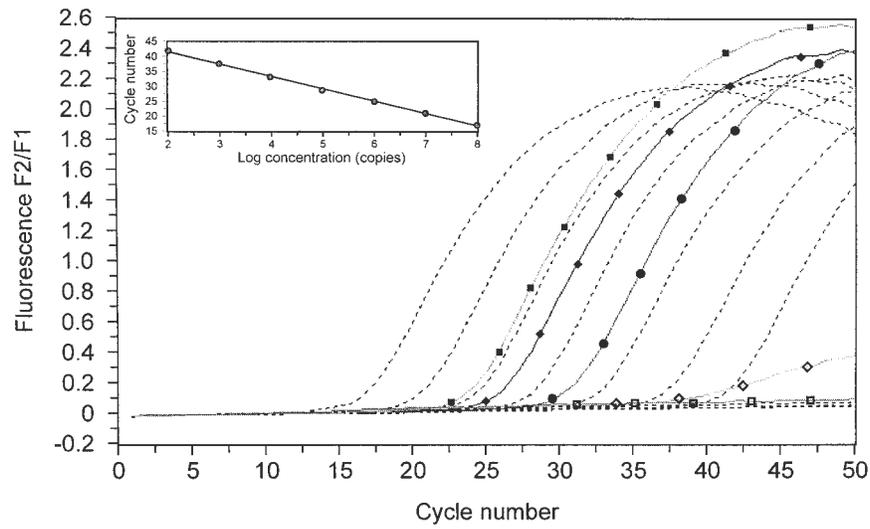
**A****B**

Fig. 1. Detection of telomerase mRNA (hTERT; **A**) and telomerase RNA component (hTR; **B**) in HeLa (◆), HEK293 (■), and HuVec (●) cells by real-time, on-line RT-PCR analysis on the LightCycler. Open symbols represent the corresponding (–RT) controls of the samples. Calculation of the copy numbers of starting templates was done as described by comparing the relative fluorescence signals of the samples to external hTERT and hTR RNA standards (broken lines). The RNA standards used were 10-fold dilutions starting from  $10^6$  copies/reaction (hTERT; **A**) and  $10^8$  copies/reaction (hTR, **B**), resulting in the standard curves shown in the upper left part of each graph. Total RNA (200 ng) of each cell line was analyzed in 5-fold replicates resulting in the following initial copy numbers: HEK293, hTERT:  $2998 \pm 130$  copies (cycle threshold point value [ $C_T$ ]:  $27.75 \pm 0.06$ ), hTR:  $1,043,000 \pm 57,270$  copies ( $C_T$ :  $24.86 \pm 0.09$ ); HeLa, hTERT:  $425 \pm 48$  copies ( $C_T$ :  $30.67 \pm 0.17$ ), hTR:  $372,100 \pm 13,430$  copies ( $C_T$ :  $26.63 \pm 0.06$ ); HuVec, hTERT:  $26 \pm 4$  copies ( $C_T$ :  $34.38 \pm 0.27$ ), hTR:  $18,500 \pm 3272$  copies ( $C_T$ :  $31.81 \pm 0.29$ ).

## 2. Materials

Unless otherwise stated, all reagents were purchased from Roche Roche Applied Science and are of the highest grade possible. All reagents were prepared using DEPC-treated H<sub>2</sub>O. For further precautions, see Note 1.

1. HighPure RNA Isolation Kit. Store at room temperature.
2. mRNA Isolation Kit for Tissue. Store at +4°C.
3. LightCycler DNA Master Hybridization probes. Store at –20°C.
4. AMV Reverse Transcriptase, 20 U/μL. Store at –20°C.
5. hTR-primers, 5 μM (see Note 2). Store at –20°C.
6. hTERT-primers, 5 μM (see Note 3). Store at –20°C.
7. Hybridization probes, 2 μM (see Note 4). Store at –20°C.
8. Reaction buffer, 1×: LightCycler DNA Master Hybridization probes, 1× (contains *Taq* DNA polymerase, dNTP mix with dUTP instead of dTTP), 1.6 U of AMV Reverse Transcriptase, 4 mM MgCl<sub>2</sub>, 0.5 μM each primer (hTR- or hTERT-specific, respectively; see Notes 5 and 6), 0.2 μM each hybridization probe (hTR- or hTERT-specific, respectively; see item 7). Prepare immediately before use and do not store.
9. T7 RNA polymerase, 20–40 U/μL. Store at –20°C.

10. T7 transcription buffer, 10×: 0.4 M Tris-HCl, 60 mM MgCl<sub>2</sub>, 100 mM dithiothreitol, 20 mM spermidine, pH 8.0). Store at –20°C.
11. Nucleotide mixture: 10 mM each ATP, CTP, GTP, and UTP. Store at –20°C.
12. RNase inhibitor, 20 U/μL. Store at –20°C.
13. hTERT standard: 5 × 10<sup>8</sup> copies/μL. The copy number was calculated based on the absorbance at 260 nm of the isolated *in vitro* RNA transcripts (See Note 5). Store at –20°C.
14. hTR standard: 5 × 10<sup>8</sup> copies/μL. The copy number was calculated based on the absorbance at 260 nm of the isolated *in vitro* RNA transcripts. See Note 5. Store at –80°C .
15. Standard dilution buffer: TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0) containing 20 ng/μL poly(rA). Store at –20°C.
16. LightCycler capillaries.
17. LightCycler instrument.

### 3. Methods

#### 3.1. Preparation of Total RNA and mRNA

1. Preparation of total RNA and mRNA was done according to the manufacturers instructions in the corresponding package insert with the following modification for the HighPure RNA Isolation Kit: The final elution step was repeated 3× by reloading the eluate to the spin column.
2. When not immediately performing the RT-PCR reaction, shock freeze the RNA/mRNA in aliquots in liquid nitrogen and store the RNA at –80°C.

#### 3.2. Preparation of RNA Standards

1. Linearize plasmids encoding hTR and hTERT DNA at the 3' end of the corresponding cDNA
2. Purify the linearized plasmids by phenol/chloroform extraction following ethanol precipitation.
3. Incubate 1 μg of linearized plasmid DNA for 2 h at 37°C in T7 transcription buffer, 1× containing 2 μg of nucleotide mix, 40 U of T7 RNA polymerase, and 20 U of RNase inhibitor.
4. After incubation, stop the reaction by heating up to 65°C for 5 min.
5. Purify the *in vitro*-transcribed RNA as described in **Subheading 3.1.**

6. Calculate the copy number of the *in vitro* transcripts based on absorbance measurements at 260 nm (1  $A_{260}$  units correspond to 40  $\mu\text{g}/\text{mL}$  RNA).
7. Dilute the isolated *in vitro* transcripts to  $5 \times 10^8$  copies/ $\mu\text{L}$  in standard dilution buffer and store the dilutions at  $-80^\circ\text{C}$ .

### 3.3. RT-PCR Amplification

The procedure described is identical for both targets, hTR and hTERT. All pipetting steps should be done on ice.

1. To prepare the reaction buffer, 1 $\times$ , transfer for each reaction 2  $\mu\text{L}$  of LightCycler DNA Master Hybridization probes, 10 $\times$ , 2.4  $\mu\text{L}$   $\text{MgCl}_2$  (25 mM), 0.1  $\mu\text{L}$  (1.6 U) AMV Reverse Transcriptase, 2  $\mu\text{L}$  of each primer, 2  $\mu\text{L}$  of each hybridization probe, and 5.5  $\mu\text{L}$   $\text{H}_2\text{O}$  into a LightCycler capillary (see Note 6).
2. As negative controls and for controlling DNA contaminations of the RNA preparations prepare the same reaction buffer containing  $\text{H}_2\text{O}$  instead of AMV Reverse Transcriptase (–RT control) for all samples to be analyzed.
3. Add 2  $\mu\text{L}$  of total RNA (50–200 ng) to the reaction buffer, 1 $\times$ . For hTERT analysis, mRNA preparations can be used instead of total RNA preparations.
4. For preparation of the standard curve, dilute the RNA standards 10-fold, ranging from  $5 \times 10^6$  to  $5 \times 10^0$  copies/ $\mu\text{L}$  in standard dilution buffer.
5. Add 2  $\mu\text{L}$  of the diluted standards to capillaries containing the reaction buffer, 1 $\times$ .
6. Quick-spin the capillaries in the corresponding adaptors in a benchtop centrifuge (2000 rpm, 30 sec).
7. Transfer the capillaries to the LightCycler instrument and perform a RT-PCR reaction by the following protocol:  $60^\circ\text{C}$  for 10 min,  $95^\circ\text{C}$  for 30 sec for one cycle each; then at  $95^\circ\text{C}$  for 0 sec,  $60^\circ\text{C}$  for 10 sec,  $72^\circ\text{C}$  for 10 sec for 50 cycles.

### 3.4. Quantification of hTR and hTERT Transcripts

Quantitative results of real-time fluorescence PCR are assessed by determination of the turning point where the fluorescence of a given sample becomes significantly different from the baseline

signal. In the LightCycler system this turning point is calculated automatically by the instrument using the second derivative maximum algorithm. Unknown copy numbers in the samples were calculated using an external hTR and/or hTERT-specific standard curve. For all RT-PCR's the correlation coefficient  $r$  was  $>0.99$ , indicating a precise log-linear relation in the range of  $10^7$  to  $10^1$  copies per reaction.

#### 4. Notes

1. To detect telomerase components by RT-PCR, there is a need for extreme caution to prevent RNase/DNase contamination that might cause degradation of the RNA template and of the amplicon. To allow achieving reliable results, the entire assay procedure must be performed under nuclease-free conditions. It is recommended using only nuclease-free solutions (e.g. DEPC, diethyl pyrocarbonate treated) stored in appropriate aliquots and stored separate from other reagents in the laboratory. To minimize the risk of carry-over contamination it is recommended to physically separate the workplaces for sample preparation and RT-PCR amplification.
2. As there is no intron–exon structure of the hTR gene, a hTR-specific primer cannot be designed to differentiate between RNA-specific PCR products and those derived from contaminating DNA in the RNA preparations. With the preparation methods used here, typically DNA contaminations of less than 0.1 % were observed.
3. Several hTERT splice variants have been demonstrated to be expressed (20). To detect only splice variants that could be translated to a putative functionally active telomerase, primers were designed to specifically detect hTERT RNA transcripts containing the  $\beta$ -region.
4. hTR- and hTERT-specific hybridization probes were designed to hybridize to adjacent internal sequences within the PCR product complementary to the sense strand of the corresponding RNA. The 5' -located probes (donor) were labeled at their 3' -ends with fluorescein; the 3' -located probes (acceptor) were labeled at their 5' -end with LightCycler- Red 640. To prevent probe extension, phosphate groups were attached to the 3' -ends of the acceptor probes.
5. The plasmids used for generation of the hTR and hTERT *in vitro* RNA transcripts contain the complete cDNAs for hTR (Accession

no. U86046) and hTERT (Accession no. AFO Gen Bank AFO15950) in a T7 vector and were provided by Geron Corporation.

6. To ensure the same reaction conditions for all samples, the samples, standards, and negative controls should be analyzed simultaneously using single master mixes for the (+RT) reactions (samples and standards) and the (–RT) controls.

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## Standard TRAP Assay

Angelika M. Burger

### 1. Introduction

The polymerase chain reaction (PCR) based telomeric repeat amplification protocol (TRAP) assay, to detect telomerase activity was originated by Kim *et al.* in late 1994 (**1**) and revolutionized the field of telomere/telomerase research in aging, but particularly in cancer. Thus, the TRAP assay has provided the stimulus for the current interest in the detection of telomerase activity in a wide variety of human malignancies with regard to its potential clinical utility, as a diagnostic adjunct in the early diagnosis of malignancy, as a potential prognostic indicator (**2–7**) or as a means to identify telomerase inhibitors (**7, 8**). Previously, investigators had to rely on the elaborate conventional primer extension assay for detecting telomerase activity, which requires large amounts of telomerase-positive cells ( $2 \times 10^8$ ), thus limiting the number of primary human specimens that could be examined easily (**9,10**). A major problem with the conventional assay is also the weak signal strength, necessitating extensive autoradiographic exposure times (more than a week). Moreover, enhancement of signal would require enrichment of the telomerase fraction and additional time consumption (**11**).

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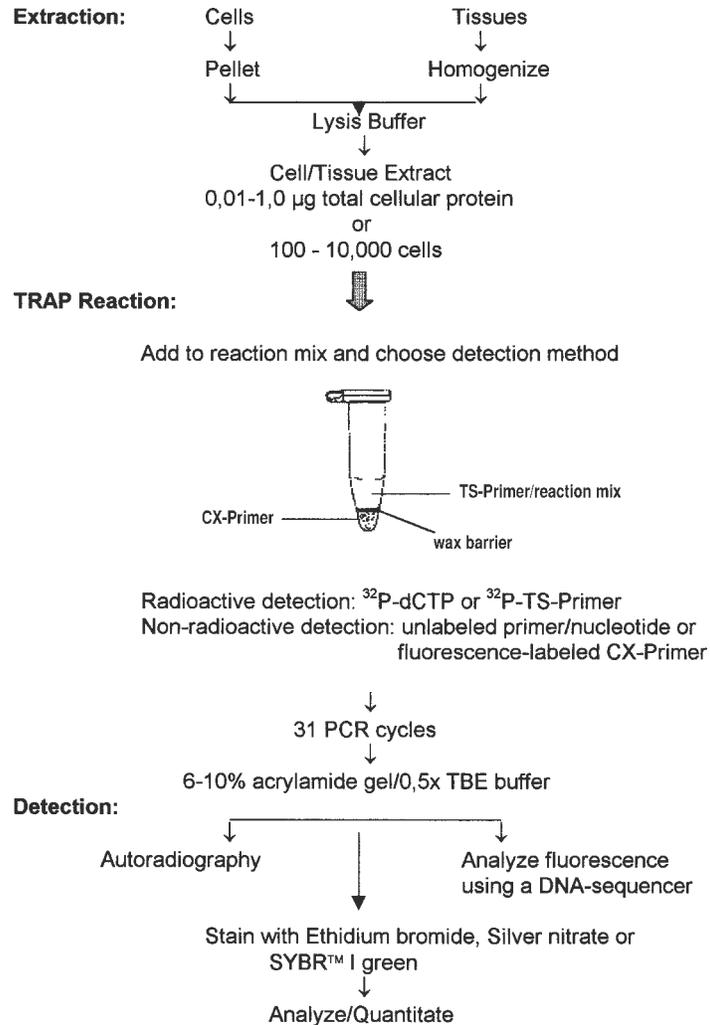


Fig. 1. Flow chart of the standard TRAP assay using a single tube reaction.

The development of the TRAP assay provided initially a highly sensitive method to measure telomerase activity owing to signal amplification by PCR and improved detergent lysis to allow more uniform extraction of telomerase from a small number of cells (1,11,12). The standard TRAP assay (Fig. 1) utilizes a single tube reaction comprised of two steps: primer extension, followed by PCR

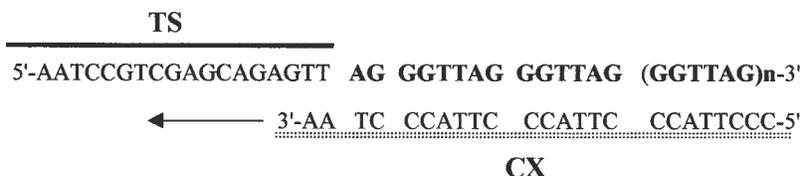


Fig. 2. Reaction 1, primer extension (TS); Reaction 2, PCR amplification (CX).

amplification of the extension products (**Fig. 2**). The TRAP assay, however, suffers also from a number of technical limitations: It can be capricious, and interpretation of results may not always be straightforward, confounded by both false-negative and false-positive results.

Detection methodology (**Fig. 1**) relies on either radiolabeling and autoradiography or staining with ethidium bromide, silver nitrate, or SYBR<sup>TM</sup> Green I and subsequent imaging for quantitation. This chapter describes the original protocols, its pitfalls, cautions, and some simplifications of the method, e.g., by using "hot-start" PCR.

## 2. Materials

### 2.1. Preparation of Telomerase Extracts from Tissues and Cell Lines

1. Washing buffer: 10 mM HEPES-KOH, pH 7.5, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 1 mM dithiothreitol (DTT), diethylpyrocarbonate (DEPC) water. The sterile filtered buffer (0.22 μm filters) can be stored at 4°C and used for 2 mo.
2. Lysis buffer: 10 mM Tris-HCl, pH 7.5, 1 mM MgCl<sub>2</sub>, 1 mM ethylene glycol-*bis*(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM β-mercaptoethanol (2-ME), 0.5% (v/v) 3-([3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate (CHAPS), 10% (v/v) glycerol, DEPC water. Prepare 10 mM Tris-HCl, pH 7.5, separately, mix all other constituents, and adjust to desired volume with the Tris-HCl buffer. Sterile filter (0.22 μm) the lysis buffer and batch out aliquots of 1–2 mL into sterile tubes. Aliquots can be stored frozen at –20°C for long periods of time. *Caution*: PMSF is extremely destructive to mucous membranes and the tissues of the respiratory tract, eyes, and skin. It may be fatal if inhaled, swallowed, or adsorbed through skin. Avoid any contact by wearing protecting clothing.

3. Bradford protein reagent: 100 mg of Coomassie brilliant blue G250, 50 mL of 95% (v/v) ethanol, 100 mL of 85% (w/v) phosphoric acid, distilled water. Vigorously stir Coomassie blue and ethanol until all dye is dissolved. Add phosphoric acid while continuing to stir for approx 1 h. Adjust volume to 1: 1 with water, store at 4°C. Solution will be good for at least 1 yr. A Bradford reagent-based assay kit is available from Bio-Rad Laboratories (Bio-Rad Protein Assay).

## **2.2. TRAP Reaction: Primer Extension and PCR Amplification**

1. CX-primer tubes: 0.1 µg CX-primer: 5'-CCCTTACCCTTACCC-TTACCCTAA-3' (custom made, HPLC-grade), Ampliwax™ gems (Perkin Elmer, Branchburg, NJ, USA). Lyophilize 0.1 µg of CX primer (dissolved in DEPC water, 0.1 µg of primer in 6 µl of H<sub>2</sub>O) onto the bottom of 0.5-mL thin-wall PCR tubes using a speed vacuum dryer (50°C). Add one wax gem per tube and melt (at 95°C in a thermocycler) to seal the CX primer layer (*see* flowchart in **Fig. 1**). Check solidified wax layer for proper sealing; no air bubbles should be visible. This is a very critical step for good performance of the assay (*see* **Note 1**). Tubes can be stored for 3 mo at 4°C.
2. PCR reaction mix, final concentration per 50 µL sample: 20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 63 mM KCl, 0.005% (v/v) Tween-20™, 1 mM EGTA, 50 µM dNTPs (Roche), 0.1 µg of TS-primer: 5'-AATCCGTCGAGCAGAGTT-3' (custom-made, HPLC-grade), 1 µg of T4g32 protein (Roche), 5 µg of BSA, 2U of *Taq* DNA polymerase (Roche), 2 µCi of (α-<sup>32</sup>P)dCTP (specific activity 3000 Ci/mmol, Amersham International) - for standard radioactive detection, DEPC-treated distilled water, tissue lysates ranging from 0.6 to 6.0 µg or cell lysates from 0.1 to 1.0 µg total cellular protein.
3. The reaction mixture should be prepared freshly from stock solutions just prior to PCR processing. Aliquot an appropriate amount into a CX primer tube and add telomerase lysate in the final step. Briefly mix, spin (10 sec, 12,000 rpm), and keep reactions at room temperature for 30 min before initiating PCR. Proper precautions for handling of radioactive materials need to be taken.

### **2.3. Detection of Telomerase Activity, Electrophoresis**

1. Polyacrylamide gel: 10% (w/v) acrylamide/bisacrylamide ratio 19:1 in 0.5× Tris borate EDTA (TBE) buffer (stock ready-made, GIBCO BRL), 0.1% (v/v) ammonium persulfate (APS), freshly prepared, 1% (v/v) *N,N,N',N'*-tetramethylethylenediamine (TEMED). Acrylamide solutions are light sensitive and should be stored at 4°C in the dark. Acrylamide is neurotoxic; therefore, caution should be exercised to avoid any skin contact. Gels are prepared just prior to use and pockets thoroughly rinsed to remove TEMED and APS residues. Otherwise, telomerase products can be degraded.
2. Sample loading buffer: 50% (v/v) glycerol, 50% (w/v) water containing, 1% (w/v) bromphenol blue, 1% (w/v) xylene cyanole FF (Sigma #4126). Use 3 µL per 50 µL of sample. The loading buffer can be stored at 4°C for several months.
3. Running buffer: 0.5× Tris-borate EDTA.

### **2.4. Internal Controls**

1. Negative controls: 500 µg/mL DNase free RNase (e.g., Roche).

### **2.5. Equipment**

1. Beckman Optima TL ultracentrifuge.
2. UV-VIS spectrophotometer.
3. Eppendorf PCR tubes.
4. Speed vacuum dryer.
5. Sterile glass tissue homogenizer tubes (e.g., Bibby, Sterilin Ltd., Stone; or Merck, Lutherworth, UK), alternatively disposable Kontes tubes and pestles are also available (Molecular Dynamics, Sunnyvale, CA).
6. PhosphorImager or densitometer.
7. Thermocycler with temperature control or heated lid.
8. A range of pipets; one pipet for very small volumes, e.g., Eppendorf Reference (0.1–2.5 µL).
9. Sterile, Biopure™ (Eppendorf) pipet tips.
10. A vertical slab gel electrophoresis unit (at least 18 × 16 cm), spacers 1.0 mm thick.
11. Gel dryer.

### 3. Methods

Telomerase is a ribonucleoprotein enzyme; therefore, RNase contamination needs to be prevented. It is advisable to wear gloves in all steps of the assay protocols, always use DEPC-treated water for preparation of solutions, and aliquot reagents in small batches and keep them stored separately. A set of separate pipets should be kept for the TRAP assay, and the working area cleared and cleaned.

#### 3.1. Telomerase Extracts

##### 3.1.1. Tissues

1. Surgical specimens should be flash frozen in liquid nitrogen immediately after removal and stored at  $-80^{\circ}\text{C}$ . If possible, prepare cryosections prior to tissue extraction to ensure viability and homogeneity. If necessary, specimens might need to be microdissected to separate regions of different pathologies. If maintained properly in liquid nitrogen or  $-80^{\circ}\text{C}$ , even tissue specimens stored for several years can be used for extraction of telomerase activity.
2. Approximately 30–50 mg of tissue is washed first in 1 mL of ice-cold phosphate buffered saline (PBS) followed by 1 mL of telomerase washing buffer. Remove traces of washing buffer as thoroughly as possible and add 250  $\mu\text{L}$  of lysis buffer.
3. Homogenize using sterile glass tissue grinders and keep homogenates on ice for 30 min.
4. Centrifuge for 30 min at 25,000g and  $4^{\circ}\text{C}$  (e.g., Beckman, refrigerated Optima TL Ultracentrifuge). Remove the resulting supernatant carefully, flash freeze, and store at  $-80^{\circ}\text{C}$ .
5. Telomerase extracts can be thawed and refrozen multiple times and are stable for several months. Total cellular protein should be determined by Bradford Assay (**13**). Multiple concentrations ranging from 6.0 to 0.06  $\mu\text{g}$  of total cellular protein should be assayed initially to determine the optimum protein concentration/saturation of the PCR reaction for the TRAP assay.

##### 3.1.2. Cell Lines

1. Cells grown in culture are trypsinized, pelleted, and the pellet washed first with ice-cold PBS and then with chilled washing buffer.

In between washing steps, cells are pelleted at 10,000g and 4°C for 1 min. Depending on the size of the cell pellet, 25–100 µL of lysis buffer (*see Note 2*) is added and cells resuspended by pipeting 3–5 times through a 200 µL tip.

2. Cells are incubated on ice for 30 min, and homogenates are centrifuged for 30 min at 100,000g and 4°C. If it is intended to quantitate telomerase activity, it is advisable to assay a range of 1.0–0.001 µg of total cellular protein extract rather than relate telomerase activity to cell number. Thus, use of cell number as a measure for activity must rely on viability of cells; no cells should be lost during the washing procedure (*see Note 3*).

### 3.1.3. Protein Estimation by Bradford Assay

The Bradford method, e.g., Bio-Rad Protein Assay (Bio-Rad Laboratories) should be used to determine protein content in cell and tissue extracts.

1. For very small samples, perform microassay version, which requires 1 mL of Bradford reagent and only 5 to 10 µL of telomerase extract.
2. Optimal protein concentrations, which just saturate the PCR reaction, but do not inhibit the *Taq* DNA polymerase, were found to be 1.0 to 0.1 µg for cell lines and 0.6 to 0.06 µg for tissues (*II, 14*).
3. Concentrated extracts can be diluted with lysis buffer or DEPC water just before addition to the reaction mixture. Telomerase lysis buffer does not interfere with the Coomassie blue-based Bradford reaction, whereas the widely used BCA Protein Assay (Pierce, Rockford, IL) (*II*) shows substantial background staining with lysis buffer (optical density [OD] about 0.5 for 5 µL of lysis buffer) caused by the buffer constituents Tris-borate and 2-ME (compare interferences listed in manufacturers instructions). This may result in false positive- and/or -negative reactions through an unreliable protein measurement. Therefore, the Coomassie blue based protein determination is the method of choice for reproducible telomerase activity measurements.

### 3.3. TRAP Reaction (Primer Extension)

1. The telomeric repeat amplification protocol (*see* flowchart in **Fig. 1**) comprises two steps in a single tube reaction (schematically shown in **Fig. 2**).

2. During the 30-min incubation period of telomerase extract with reaction mixture (50  $\mu$ L/tube), a nontelomeric synthetic oligonucleotide (TS, forward primer), which contains the telomeric repeat sequence recognized by telomerase, serves as artificial telomere. If telomerase is present in the extract, it will add further telomeric repeats onto the 3' end of the TS oligonucleotide. Thus, it is important for the primer extension reaction that it is separated from the reverse primer and active *Taq* DNA polymerase. Therefore, TS and CX primers are either separated by a tightly sealed wax barrier (*see Fig. 1*) or conventional *Taq* DNA-polymerase must be substituted by a heat-activated hot-start *Taq* polymerase (*see Note 4*).

### 3.4. PCR Amplification

1. In the second step, another oligonucleotide is introduced that is complementary to the repeat sequence so that the extended synthetic oligonucleotides are then amplified by PCR (**Fig. 2**). The amplification reaction is initiated by incubation of samples at 90°C for 90 s. This inactivates telomerase and releases the CX primer by melting the wax barrier (melting point of Ampliwax = 70°C).
2. Telomerase products are amplified in 31 PCR cycles: 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s and held at 4°C. If kept at 4°C, samples are stable for several days.

### 3.5. Detection of Telomerase Activity

1. The whole PCR mixture (50  $\mu$ L) is analyzed on 10% nondenaturing, acrylamide gels. To facilitate monitoring and loading of the samples, 3  $\mu$ L of loading buffer is added.
2. The acrylamide mixture should be filtered before addition of TEMED, and the polymerized gel is run in 0.5 $\times$  Tris-borate, pH 8.3, EDTA buffer at 155 V for 5–6 hr to obtain good resolution of banding pattern. At this point, the xylene cyanole front should be approx 3–5 cm from the bottom of the gel.
3. The anode tank buffer is highly radioactive and needs to be handled and disposed of with all precautions that apply for radioactive waste.
4. The gel is blotted onto filter paper (Whatman 3MM), covered with Saran Wrap, and dried directly (80°C for 1 h).
5. Gels are then either exposed to a sensitive film (e.g., Kodak, X-Omat AR) for 24 h or analyzed using a PhosphorImager, if available (*see*

**Note 5).** To confirm negative results, one might extend the exposure time up to 3 days.

6. The amplified telomerase products are of heterogeneous length and create a typical, regular ladder pattern of bands, each representing the addition of a hexanucleotide (TTAGGG) telomeric repeat by telomerase (**Fig. 3A**). Quantification of telomerase activity can be attempted if multiple telomerase extract concentrations are assayed from each sample and compared within the same set of probes (**Fig. 3A**).
7. Autoradiographs may be quantified using scanning gel densitometry (e.g., with a Sharp JX 330P scanner and Phoretix 1D software, Phoretix International). A defined number of clearly visible bands for each TRAP reaction should be chosen, and their mean signal intensity calculated (**Fig. 3B,C**).

### 3.6. Internal Controls

1. False-negative and false-positive reactions are inherent problems of the standard TRAP assay (*see Note 1*). Authenticity of the telomerase ladder signal can be confirmed by assaying in parallel RNase-treated lysates. RNase will destroy the essential part of telomerase enzyme activity, the RNA component, and so disable the telomeric TTAGGG repeat synthesis. On the contrary, false-positive ladder signals will be insensitive to RNase pretreatment.
2. Telomerase lysates (approx 1  $\mu$ L) are mixed 1:1 with DNase-free RNase 30 min (room temperature) before addition of the appropriate extract quantity to the PCR reaction mixture.
3. The possibility of false-negative samples can be eliminated by the addition of internal standards (*see Note 1*) or by mixing "negative" samples with confirmed positive extracts in repeat experiments.
4. It is generally advisable to run a known, telomerase-positive sample and a lysis buffer blank as controls for proper PCR assay performance with each set of experiments (*see Notes 5–7*).

### 4. Notes

1. False-negative and -positive reactions are the major pitfalls of the standard TRAP assay. **Figure 4A** shows examples of false-positive reactions. The depicted samples do not contain telomerase extracts and are solely caused by TS-CX primer dimer artifacts. The artifacts occur if TS and CX primers are mixed in the presence of conventional

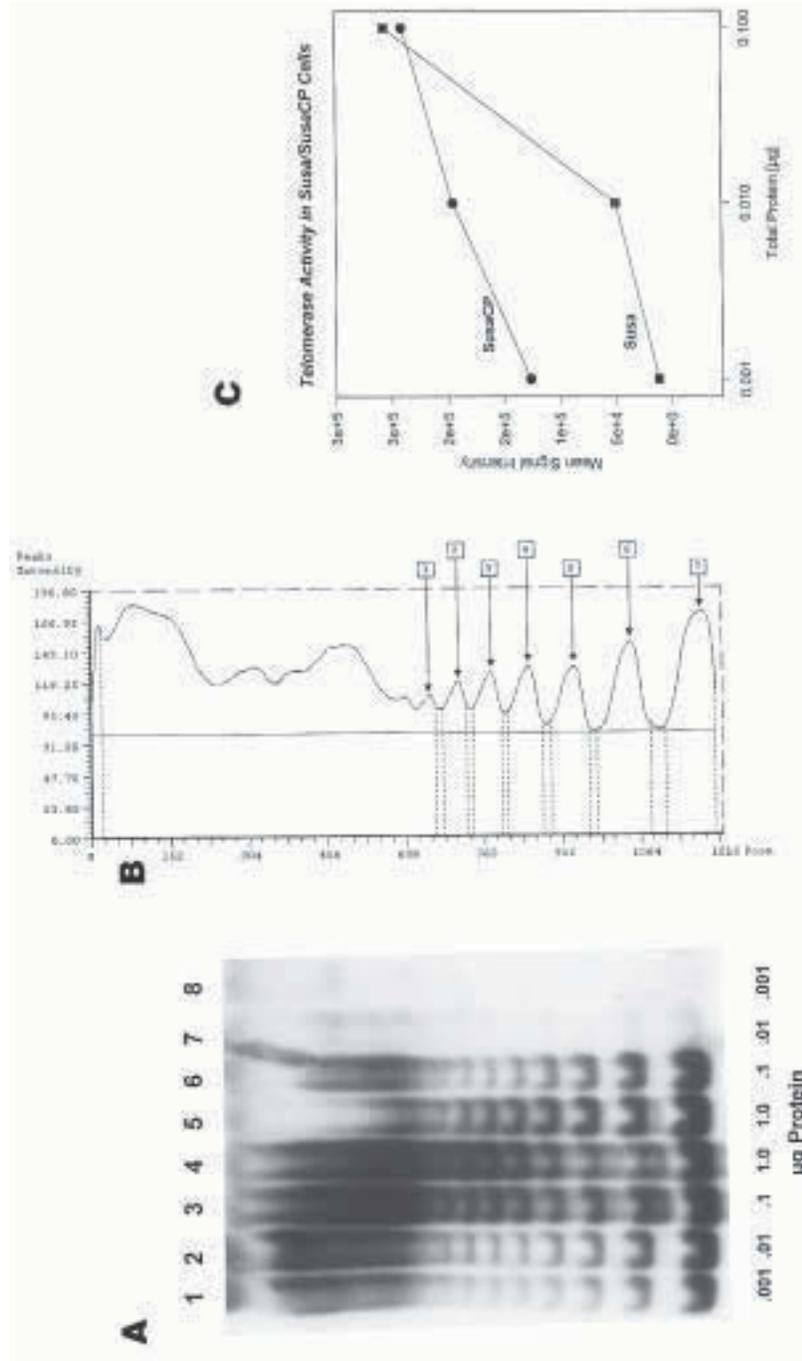


Fig. 3.

*Taq* DNA polymerase either by omission or by the presence of a wax barrier that has not been sealed properly. The false-positive result on the autoradiograph is characterized by the random variation in band intensity and the absence of progression of bands close to the origin (**Fig. 4A**). If it is unclear whether a reaction is false positive, samples need to be reassayed, including a RNase digestion control. False-positive reactions occur despite the presence of RNase and are mainly a result of the leakage of CX primer tubes. The smallest hexanucleotide repeat fragment seen (**Fig. 4B**, arrow) is the most intense band of a telomerase ladder with an approximate size of 40 bp. The 40-bp fragment is also the theoretical size of TS-CX primer dimer formation/artifacts. In some instances, this band might be the only one present. If so, it should be only considered a genuine telomerase signal if it can be abolished by RNase digestion in subsequent repeat reactions. Another phenomenon within the banding pattern of a telomerase signal is the appearance of "double bands," which are faint bands approx 2 bases below the regularly spaced intense hexanucleotide repeats (**Fig. 4B**, asterisk). They belong to a genuine telomerase signal and are the result of primer slippage within repeat sequences. False-negative reactions can either be caused through inhibition of the *Taq* DNA polymerase by high protein concentrations in cell extracts or the absence of protein resulting from loss of the cell pellet during processing. Thus, the determination and titration/dilution of the cellular protein concentration of each sample

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Fig. 3. (opposite page) Typical appearance and quantification of TRAP assay signals. (A) Telomerase activity in the parental Susa (lanes 5–8) and the cisplatin-resistant SusaCP (lanes 1–4) testicular cancer cell lines. Shown are assays of a range of total cellular protein (0.001–1.0  $\mu\text{g}$ ) for each cell line. Susa/SusaCP cells produce intense telomerase ladders of high processivity. (B) Exemplary densitometric evaluation of A (lane 6). Depicted is the peak intensity of the distinguishable hexanucleotide repeats within this lane. (C) Comparison of telomerase activity in Susa and SusaCP cells based on the determination of the mean signal intensity of six successive repeats. Enzyme activity in 0.001, 0.01, and 0.1  $\mu\text{g}$  of protein for each cell line is shown. The parental Susa cell line has less telomerase than the cisplatin-resistant SusaCP cells. This figure clearly demonstrates the need for dilution of telomerase activity to enable quantitation of PCR-amplified products.

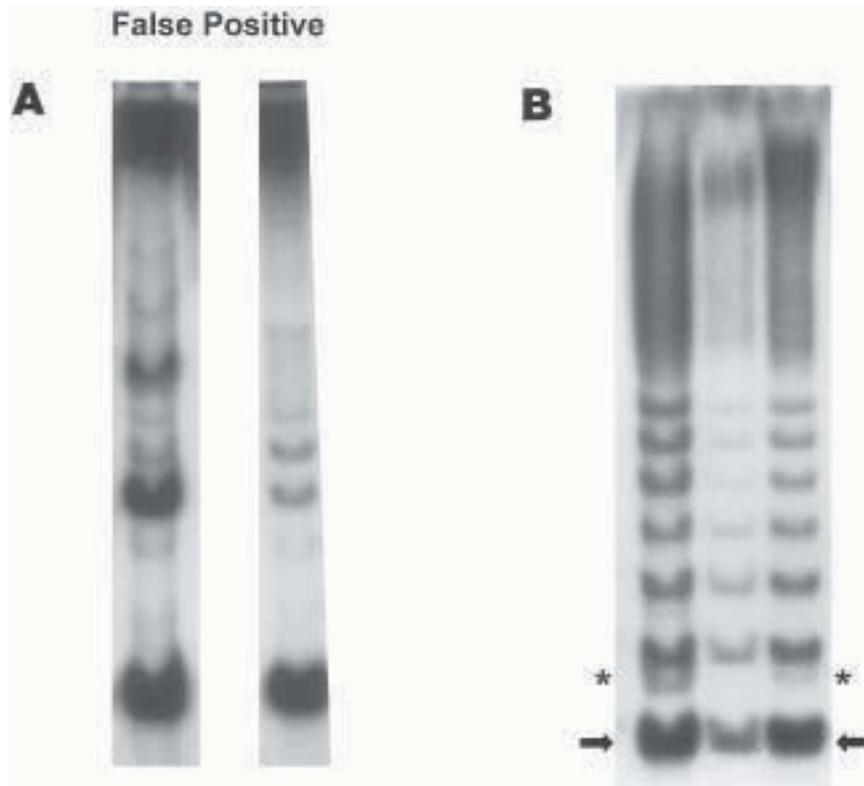


Fig. 4. (A) *False-positive TRAP assay reactions*. TS-CX primer dimer formation can mimic the presence of telomerase activity in absence of cell extract. This phenomenon often occurs if the wax barrier, separating the CX primer from the TRAP reaction during TS primer extension, is damaged. A false-positive result is characterized by random variation in band intensity and absence of regular banding pattern close to the origin. (B) *Primer slippage*. Shown are genuine telomerase repeat signals with double bands indicated by an asterisk (\*). These bands are the result of primer slippage, a problem inherent in repeat sequences. Arrows mark the 40-bp origin of the TRAP assay amplification products.

should be obligatory. Attention to these methodological details allows for quantitative comparison to be made between results obtained from consecutive experiments within the same laboratory (Fig. 3A). To prevent the occurrence of false-negative reaction, it might be useful to include the internal telomerase assay standard

(ITAS). ITAS was developed by Wright et al. (18) to provide an external quality assurance mechanism and enable better linearization of the TRAP assay. It is a myogenin cDNA fragment containing TS and CX primer sequences and is therefore amplified during the TRAP reaction to a 150-bp product. The product should occur independently of the presence of telomerase activity.

2. A modification of the original lysis buffer described by Kim et al. (1) recently has been reported to enhance the extraction efficiency of telomerase activity from cell lines. Through addition of 1% NP-40 and 0.25 mM deoxycholic acid to the lysis buffer constituents (**Sub-heading 2.1., item 2**), it is now possible to reextract a cell pellet with the improved buffer to obtain more/additional enzyme activity (15). This can be of particular use if very small quantities of biopsy material (e.g., fine needle aspirates) and/or precious, slow-growing primary cell cultures are examined. More extract in these cases would then allow for repeat assays.
3. From our experience in the handling of cultured cells for extraction of telomerase activity, the total number of 100,000 cells appears to be the lower limit. Cell pellets of less than 100,000 cells are not only barely visible, but also difficult to quantify. If counted prior to washing, there will always be a substantial loss during processing. Also, the protein content of such a small cell number will be so low that it cannot be determined with the available methodology (Bradford assay). If the number of available cells is limited, an *in situ* telomerase assay should be used (see Chapter 11).
4. The standard TRAP reaction requires the initial separation of the CX primer by a wax barrier from the reaction mixture during the TS primer extension step. This involves elaborate and time-consuming processes for preparation of CX primer tubes. Thus, lyophilization and sealing of the CX primer can be omitted and the whole procedure greatly simplified if a heat-activated *Taq* DNA-polymerase such as AmpliTaq Gold™ (Perkin Elmer, Applied Biosystems) (16) is used. Furthermore, it is even possible to use AmpliTaq Gold in conjunction with the supplied reaction buffer by simply adding dNTPs, TS, and CX primers. Then there is no further need to add T4g32 protein (initially used for unspecific background reduction). AmpliTaq Gold is activated by heat treatment for 10 min at 95°C prior to PCR cycling, which allows a simple hot-start PCR procedure after the 30-min telomerase primer extension reaction. The

modifications described greatly decrease the cost of the TRAP assay without loss of quality.

5. It is also possible to detect the telomerase ladder signal directly by using silver stain, SYBR Green I, or ethidium bromide (*see Fig. 1*). The radioactive detection with [<sup>32</sup>P] dCTP incorporation, however, is the most sensitive method and should be used if weak telomerase activity is expected, e.g., in several normal tissues/cells with low intrinsic enzyme activity such as activated peripheral blood leukocytes. Silver staining is performed after a 30-min fixation of the gel in 10% acetic acid followed by three rinses with water and a 45-min incubation in AgNO<sub>3</sub> solution before developing (Daiichi Pure Chemicals 2 D-Silver Stain II kit; Daiichi, Tokyo, Japan). SYBR Green I is used as 10,000× diluted stock as provided by the manufacturer (Molecular Probes, Eugene, OR, USA) in 50 mM Tris-HCl (pH 8.0), and gels immersed in this solution for 45 min. Ethidium bromide is applied according to standard procedures used for agarose gels (*17*).
6. Commercially available kits based on the TRAP assay for detection of telomerase activity have now become accessible. The TRAP-eze™ Telomerase Detection Kit is distributed by Oncor (Gaithersburg, MD) and provides lysis buffer and most PCR reaction components of the standard TRAP assay (*1*). The benefit of this kit is that it includes an internal PCR control (size approx 30 bp), which helps to eliminate false-negative reactions (*see Note 1*). A second kit is offered by Boehringer Mannheim (Germany), the Telomerase PCR Elisa™. This TRAP assay variant is described in detail in Chapter 10.
7. Given that all cautions described above are considered and that for interpretation of TRAP assay results attention is paid to the possibility of false-positive reactions, the standard TRAP assay still provides a very sensitive, fast, and remarkably reproducible method to determine telomerase activity in fresh cell and tissue materials.

## Acknowledgments

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## Stretch PCR Assay

Jun-ichi Nakayama and Fuyuki Ishikawa

### 1. Introduction

In 1985, telomerase activity was identified in the macronuclei of the ciliate *Tetrahymena* and was found to add telomeric repeats onto telomeric oligonucleotide primers (1). The radiolabeled elongated products were detected easily as a 6-bp ladder on an electrophoresis gel. In 1989, using the same method, human telomerase activity was identified in a HeLa cell extract (2). However, the telomerase activity of human cells was very weak, and it was difficult to analyze the biochemical properties of telomerase or to investigate the significance of telomerase in various diseases, such as cancer. Therefore, we began to investigate a sensitive polymerase chain reaction (PCR)-based detection method for telomerase activity. Our goal was to develop a method that was not only versatile but also useful for biochemical analyzes, such as the measurement of the level of processing and the kinetics of telomerase.

Telomerase produces telomeric DNA composed of telomeric repeats. There were two major problems to overcome in the amplification of the telomerase product. First, any combination of forward and reverse PCR primers for repetitive sequences would anneal efficiently by themselves. As a result, short PCR products

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from primer dimers are predominantly amplified. Second, these primers can anneal with any repeat unit within the repetitive telomerase product. Therefore, if a short PCR product is formed from a primer annealed with an internal repeat of a template, the length of the PCR product does not represent the original length of the telomerase product. In 1994, the PCR-based telomeric repeat amplification protocol (TRAP) assay was reported, and with this method, telomerase was shown to be active in germ-line cells and most cancer cells, but not in normal somatic cells (3). In the TRAP assay, a non telomeric oligonucleotide primer is used as a substrate for telomerase, and mutations are introduced into the reverse primer to reduce the formation of primer dimers. To determine the activation stage of telomerase during tumorigenesis or to examine the biochemical features of the enzyme complex, it is important to quantify the telomerase activity reproducibly. However, we and other researchers have experienced some difficulty in quantifying the telomerase activity by means of the TRAP assay (4–6).

We have developed a new PCR-based method for measuring telomerase activity (7). To overcome the problems described earlier, primer sequences are carefully designed and the reaction conditions explored. In brief, the primer–primer complex formed between the forward and reverse PCR primers has 1-bp mismatch at the 3' ends, preventing the production of PCR products as primer dimers. Second, unrelated extra tag sequences are introduced onto the 5' end of each primer, maximizing the chance that PCR products represent the length of the original telomerase products. The resulting PCR protocol, designated as stretch PCR assay, is highly suitable for the measurement of relative telomerase activity (*see Fig. 1*).

**Figures 2 and 3** represent typical experiments with S100 extracts of HeLa cells. When the PCR products were analyzed by denaturing polyacrylamide gel electrophoresis, telomerase activity was represented as a 6-base ladder (**Fig. 2A,B**). This band pattern was not observed when the extract was pretreated with RNase A, which is the standard criterion for telomerase activity (**Fig. 2A**). These signals were also not observed when the extract was treated with proteinase A or heat (data not shown). More importantly, when the

telomerase reaction was sampled at each time point, we observed that the amplified products had increased in length between 0 and 30 min of incubation, indicating that the stretch PCR assay reproduced the original telomerase products faithfully (**Fig. 2B**). As shown in **Figure 1**, the tag sequence of each primer enables the maintenance, i.e., stretch, of the PCR product. This feature permits quantitative analysis of telomerase activity. **Figure 3** shows the analysis of an S100 extract derived from  $1 \times 10^5$  HeLa cells and five-fold serial dilution. The amount of radioactivity in each lane was measured, and the quantified PCR products were plotted against cell numbers (**Fig. 3B**). A good dose response relationship was observed with extracts containing the equivalent of between 32 and  $2 \times 10^4$  cells. These results demonstrate that the stretch PCR assay detects the authentic telomerase product and is an extremely powerful tool for the measurement of telomerase activity (**8, 9**).

## 2. Materials

### 2.1. Preparation of S100 Extract

1. Phosphate buffered saline (PBS): prepare by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of  $\text{Na}_2\text{HPO}_4$ , and 0.2 g of  $\text{KH}_2\text{PO}_4$  in 1 L of distilled  $\text{H}_2\text{O}$ . Autoclave for 15 min using the liquid cycle. Cool and store at  $4^\circ\text{C}$ .
2. Cell lysis buffer: 10 mM HEPES, pH 8.0, 3 mM KCl, 1 mM  $\text{MgCl}_2$ , 1 mM EGTA, 0.5% CHAPS, 1 mM DTT, 0.1 mM PMSF, 1  $\mu\text{g}/\text{mL}$  leupeptin, 2  $\mu\text{g}/\text{mL}$  pepstatinA, 10 U/mL RNasin.
3. 5M NaCl.
4. Glycerol.
5. Liquid nitrogen.

### 2.2. Stretch PCR

#### 2.2.1. Primer Extension by Telomerase

1. 0.5 M Tris-potassium acetate, pH 8.5: Prepare by dissolving 6.06 g of Tris base and 4.91 g of potassium acetate to 80 mL of sterile  $\text{H}_2\text{O}$ . Adjust the pH 8.5 with glacial acetic acid and bring final volume to 100 mL.
2. 2 $\times$  reaction mixture: 100 mM Tris-potassium acetate, pH 8.5, 1 mM dATP, 1 mM dGTP, 1 mM TTP, 10 mM  $\beta$ -mercaptoethanol, 2 mM

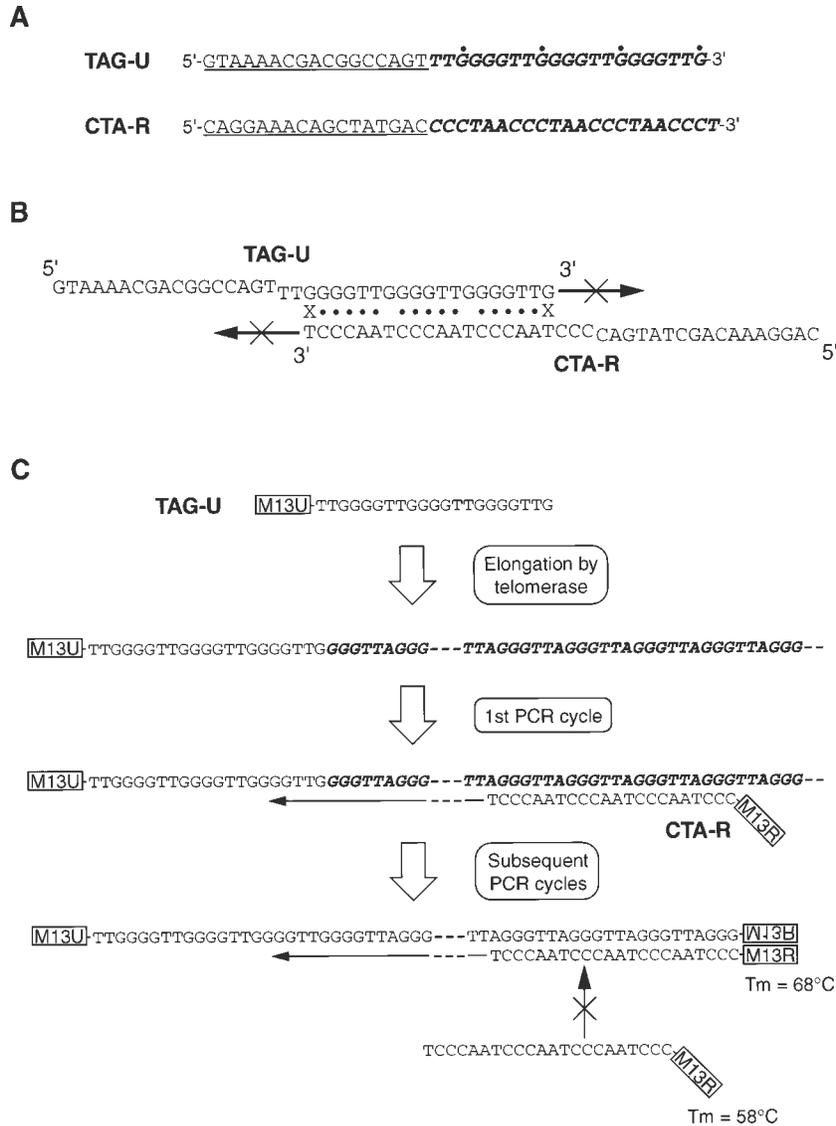


Fig. 1. Schematic diagram of stretch PCR for the detection of telomerase activity. (A) Primer sequences. The 3' half of the substrate oligonucleotide for the telomerase reaction, TAG-U, is a 21-base sequence of *Tetrahymena* telomeric TTGGGG repeats instead of human TTAGGG repeats (italics). The 5' half of the TAG-U oligonucleotide is a 17-base sequence unrelated to the telomeric sequence (M13 universe primer sequence with M13-U underlined). Human telomerase has been shown to initiate the elongation

MgCl<sub>2</sub>, 2 mM EGTA, 2 mM spermidine, 0.2 mM spermine, 1 μM denatured TAG-U primer (5'-GTAAAACGACGGCCAGTTTGGGG-TTGGGGTTGGGGTTG-3').

3. RNase mixture: 10 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.1 mg/mL RNase A.
4. Proteinase mixture: 10 mM Tris-HCl, pH 8.0, 0.5% SDS, 0.3 mg/mL proteinase K.
5. Phenol/chloroform: 1:1 (v/v).
6. Ethanol.
7. 10M ammonium acetate.
8. Yeast tRNA (Boehringer Mannheim).
9. 70% ethanol.

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(caption continued)

reaction with a variety of mutant telomeric repeats, including TTGGGG repeats (2). The 3' half of the reverse (deoxycytidine [dC]-rich) primer, CTA-R, is a 22-base tract of dC-rich human telomeric CCCTAA repeats (*italics*) and the 5' half is a 17-base M13 reverse primer (M13-R underlined). (B) As TAG-U and CTA-R are designed to have a 1-bp mismatch at both 3' ends, no product is formed from this primer-dimer complex under the stringent annealing conditions in this assay. (C) TAG-U is incubated with S100 cell extract, and telomerase adds telomeric repeats to the 3' end of the oligonucleotide. After the elongation reaction, the DNA is purified and subjected to an amplification reaction. The unreacted TAG-U primer in the reaction mixture is used as the forward primer. A reverse telomeric primer, CTA-R, is added, and PCR is performed in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP. The TAG-U primer always recognizes the 3' end of the dC-rich template DNA because of its mutant telomeric sequences. In contrast, in the first cycle of PCR, CTA-R primers anneal at any of the telomeric repeats synthesized by telomerase. However, during subsequent cycles, the 3' end of the deoxyguanosine (dG)-rich template DNA base-pairs with CTA-R over a region of 39 bases, including the M13-R sequence (the calculated melting temperature is 68°C), whereas base-pairing between CTA-R and internal telomeric repeats occurs over a region of 22 bases without the M13-R sequence (the calculated melting temperature is 58°C). Because of the difference in melting temperature between the two possible annealing sites, the CTA-R primer was expected to preferentially anneal to the terminal site at 68°C. Thus, the length of the telomerase product is maintained during the amplification step.

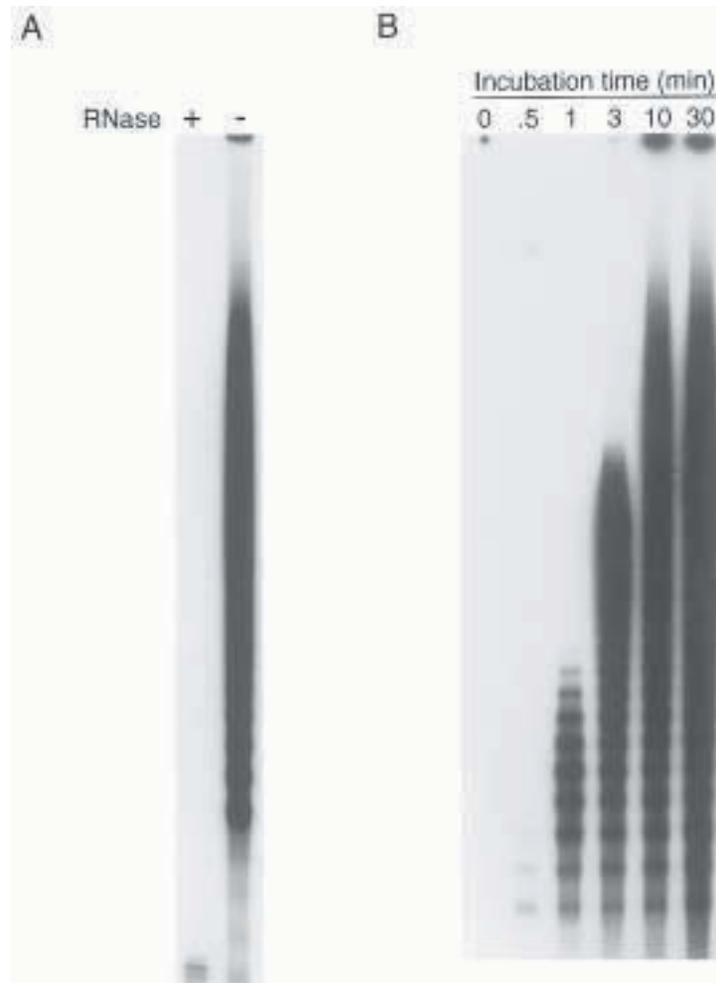


Fig. 2. Detection of telomerase products by stretch PCR. A S100 extract from  $1 \times 10^4$  HeLa cells was used for the assay. The amplified products were analyzed by electrophoresis on a 7% denaturing polyacrylamide gel. S100 extract was pretreated with RNase A (left lane, +) or mock treated (right lane, -). Since telomerase contains an RNA component as a template, the reaction is sensitive to RNase A treatment. The length of the PCR product varies after various incubation times with telomerase. S100 samples derived from  $2 \times 10^4$  HeLa cells were used in this assay. The reaction was stopped at each time point and then subjected to a further amplification step. The time course of primer elongation by telomerase was between 0 and 30 min.

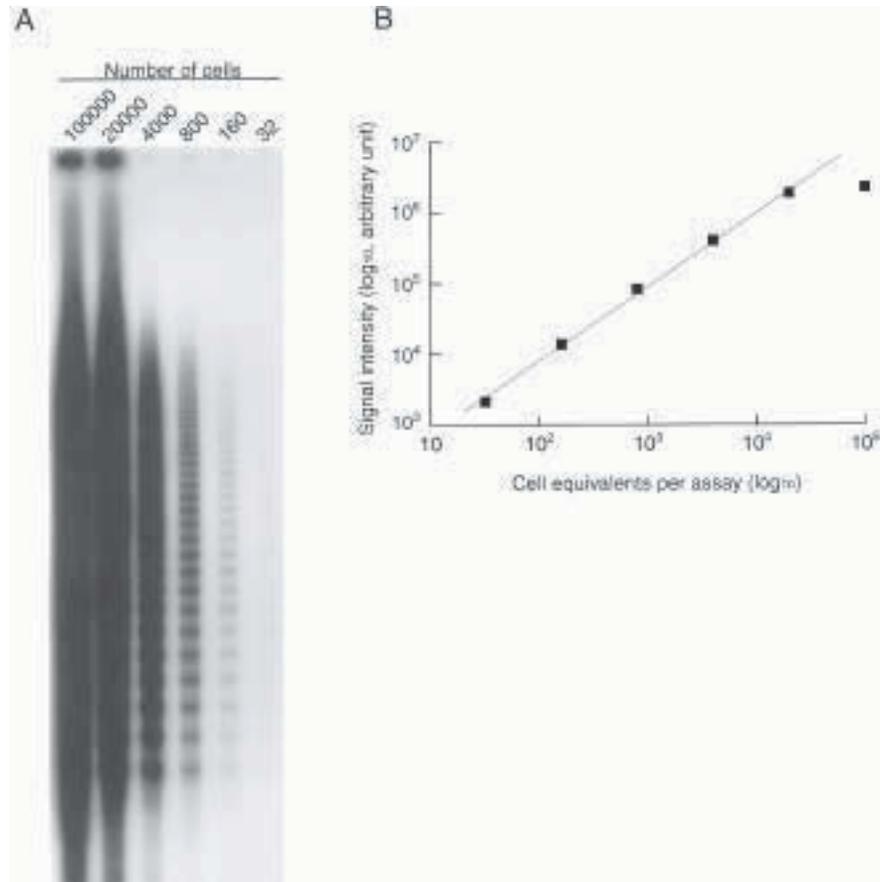


Fig. 3. Quantitative detection of telomerase activity by stretch PCR. (A) A serial 5-fold dilution of S100 extract, ranging from the equivalent of 32 to  $1 \times 10^5$  cells, was analyzed by stretch PCR assay. The amount of PCR product was quantified using a PhosphorImager (BAS200) and plotted (B) as arbitrary units (solid square). The graph was linear up to a cell equivalent of  $2 \times 10^4$  cells.

### 2.2.2. Stretch PCR Reaction

1. 10× PCR buffer: 200 mM Tris-HCl, pH 8.3, 750 mM KCl.
2. 50 mM MgCl<sub>2</sub>.
3. 1% (w/v) polyoxyethylene ether W-1 (Sigma).

4. 2.5  $\mu$ M CTA-R primer (5'-CAGGAAACAGCTATGACCCCTAACCCCTAACCCCTAACCCCT-3').
5. dNTP mixture: 10 mM dATP, 10 mM dGTP, 10 mM TTP, 1 mM dCTP.
6. *Taq* DNA polymerase (5 U/mL).
7. [ $\alpha$ - $^{32}$ P]dCTP: specific activity of 3000 Ci/mmol.
8. Mineral oil.
9. Thermal cycler (MJ Research, PTC-100).
10. Chloroform.
11. 3M sodium acetate, pH 5.2.
12. Ethanol.
13. 70% ethanol.

### 2.2.3. Gel Electrophoresis and Analysis of PCR Products.

1. Formamide loading buffer: 50% formamide, 5 mM EDTA, pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanol.
2. 10 $\times$  TBE: 0.89 M Tris base, 0.89M boric acid, 20 mM EDTA, pH 8.0.
3. 40% acrylamide stock: acrylamide/bisacrylamide, 19:1.
4. Urea.
5. 10% ammonium sulfate, stored in the dark at 4°C (stable for 1 wk).
6. TEMED.
7. Whatman 3MM paper.
8. BAS2000 (Fuji Film).

## 3. Methods

### 3.1. Preparation of S100 Extract (see Note 1)

1. Harvest the cultured cells by centrifugation for 10 min at 2000 rpm (700g max).
2. Wash twice with ice-cold PBS.
3. Resuspend the pelleted cells in cell lysis buffer at a concentration of  $2 \times 10^7$  cells/mL (Notes 1 and 2).
4. Store on ice for 20 min.
5. Centrifuge the suspension at 10,000 rpm (12,000g max.) for 10 min at 4°C.

6. Collect the supernatant and add 0.02 vol of 5M NaCl.
7. Incubate the extract at 4°C for 20 min with gentle shaking.
8. Centrifuge the extract at 40,000 rpm (100,000g max.) for 60 min at 4°C.
9. Collect the supernatant and add 0.25 vol of glycerol.
10. Divide into aliquots, freeze in liquid nitrogen and store at -80°C.

### 3.2. Stretch PCR

#### 3.2.1 Primer Extension by Telomerase

1. Add 20µL of 2× reaction mixture (*see Note 3*) to 20 µL of S100 extract.
2. Incubate the mixture at 30°C for 60 min.
3. Stop the reaction by adding 50 µL of RNase mixture. Incubate at 37°C for 10 min.
4. Add 50 µL of proteinase mixture and incubate further at 37°C for 10 min.
5. Extract with an equal volume of phenol/choroform.
6. Add 45 µL of 10M ammonium acetate, 5 µg of yeast tRNA, and 540 µL of ethanol (*see Notes 4–6*).
7. Store at -20°C for 15 min and centrifuge at 15,000 rpm (19,000g) for 15 min at 4°C.
8. Wash pellets with 70% ethanol and dry in a centrifugal evaporator.

#### 3.2.2. Stretch PCR Reaction

1. Prepare PCR reaction buffer as follows: 5.0 µL of 10× reaction buffer, 1.5 µL of 50 mM MgCl<sub>2</sub>, and 2.5 µL of 1% W-1. Add distilled water to a final volume of 47 µL.
2. Dissolve the pelleted samples in 38 µL of PCR reaction buffer and transfer to new 0.5-mL microtubes (template mixture).
3. Add 2 µL of 2.5 µM CTA-R primer to the template mixture and overlay 20 µL of mineral oil (*see Note 7*).
4. Prepare *Taq* mixture as follows: 9.0 µ L of PCR reaction buffer, 0.2 µL of dNTP mixture, 0.3 µL of *Taq* polymerase (5 U/µL), and 0.5 µL of [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol).
5. Place the tubes of template mixture into the thermal cycler, incubate at 95°C for 5 min, and cool to 80°C.

6. Incubate the *Taq* mixture at 80°C for 1 min and add to template mixture (see **Note 8**).
7. Start the amplification cycle as follows: 25 main cycles of 93°C for 1 min, 68°C for 1 min, and 72°C for 10 min, followed by a final extension of 72°C for 10 min.
8. Extract with 50  $\mu$ L of chloroform.
9. Add 0.1 vol of 3M sodium acetate (pH 5.2) and 2.5 vol of ethanol.
10. Store at -20°C for 15 min and centrifuge at 15,000 rpm (19,000g) for 15 min at 4°C.
11. Rinse pellets with 70% ethanol and air-dry.

### 3.2.3. Gel Electrophoresis and Analysis of PCR Products

1. Dissolve pellets in 2  $\mu$ L of formamide loading buffer.
2. Heat samples to 95°C for 2 min prior to loading.
3. Use a gel that is 0.35 mm thick and 20  $\times$  40 cm in size, consisting of 7% polyacrylamide and 7M urea in 1 $\times$  TBE. Prepare the gel by mixing 8.75 mL of 40% acrylamide stock, 21.02 g of urea, and 5mL of 10 $\times$  TBE. Add distilled water to a final volume of 50 mL. Filter through a 0.45- $\mu$ m filter, degas, and add 200  $\mu$ L of 10% ammonium sulfate and 40  $\mu$ L of TEMED.
4. Prerun and run the gel at a constant current of 30 mA until the bromophenol blue reaches the bottom.
5. After the run, transfer the gel to Whatman 3MM paper that has been cut to the same size as the gel, cover with Saran Wrap,<sup>TM</sup> and dry on a gel dryer.
6. Expose the gel to X-ray film or analyze using a PhosphoImager BAS2000.

## 4. Notes

1. As telomerase is a large ribonucleoprotein complex that contains an RNA component as a template, the isolation of the cell extract should be carried out at low temperature (0–4 °C) and all reagents should be free of RNase contamination. The S100 extract is stable for at least 6 mo.
2. The procedure for preparing S100 extract is suitable for a small quantity of cultured cells or for tissue samples of up to 1 $\times$ 10<sup>8</sup> cells. A

Dounce homogenizer is useful for disrupting the cells in scaled-up experiments.

3. The TAG-U primer used as a substrate for telomerase is denatured at 72°C for 5 min prior to use. This step prevents the G-rich oligonucleotides from forming the tertiary G-quartet structure.
4. The ethanol precipitation of the elongated product before the PCR amplification step is important for quantitative analysis because it has been reported that there are inhibitors of *Taq* polymerase in the S100 extract.
5. Ammonium acetate precipitation removes unincorporated nucleotides.
6. Artificial bands of high molecular weight can be amplified in the PCR step when *Escherichia coli* tRNA is used in place of yeast tRNA. Yeast tRNA is recommended in this step.
7. Although 5 pmol of CTA-R is used as the reverse primer in this PCR reaction, one-tenth of this amount of primer is sufficient to detect strong telomerase activity, such as that in cell lines or tumor samples. Increased amounts of CTA-R increases the sensitivity of this assay; however, the length of the amplified products becomes shorter at the same time.
8. To reduce the amplification of unrelated products, we recommend the use of a hot-start PCR protocol by adding *Taq* polymerase at 80°C.

## Acknowledgments

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## Fluorescent Detection of Telomerase Activity

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Mary J. DeHart, and Katherine H. Moore

### 1. Introduction

Telomerase is now a significant factor in the fight against cancer and antiaging (**1**). It is becoming a valuable prognostic tool to determine if a particular tissue is likely to develop cancer (**2**). The ability to detect telomerase activity in patients with cancer has also been correlated with clinical staging of some tumors (**3–7**). The regulation of telomerase and the ability to inhibit the enzyme is also an important aspect in future drug development for both anticancer and antiaging purposes (**8–11**). Thus, the most sensitive and accurate methods are needed to detect telomerase in various sample formats.

Original methods for telomerase detection were slow, cumbersome, and based on incorporation of specific radioactive dNTPs in the telomeric repeats added onto the 3' end of a synthetic primer (**12**). More recently, an improved detection method, the telomere repeat amplification protocol (TRAP) assay was introduced by Kim et al. (**13**). The TRAP assay increased the speed of detection and dramatically improved sensitivity by a factor of  $10^4$ . The TRAP assay is basically a two-step process. In the first step, a viable telomerase artificially extends polymeric repeats onto the 3' end of a synthetic

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forward primer, TS. This oligonucleotide facilitates extension by telomerase to generate telomeric product. The second step is PCR amplification with the addition of a reverse primer to amplify the ladder with 6-bp increments  $[\text{GGGTTA}]_n$  for detection of human telomerase.

With the inception of the TRAP assay, variations have now been added for further improvement to increase sensitivity and speed of detection as well as aid in quantification of enzyme activity

## 2. Materials

### 2.1. Preparation of Sample Extracts

1. PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM  $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ , 1.4 mM  $\text{KH}_2\text{PO}_4$ , pH 7.2.
2. Protein lysis buffer: 0.5% 3-([3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate (CHAPS) (w/v), 10 mM Tris-HCl (pH 7.5), 1 mM  $\text{MgCl}_2$ , 1 mM EGTA, 5 mM ( $\beta$ -mercaptoethanol, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), 10% glycerol (v/v). Keep frozen in small aliquots to eliminate repeated freeze-thaw cycles, which degrades some components ( $\beta$ -mercaptoethanol is a volatile reagent).
3. Homogenization tubes with matching pellet/pestle for grinding up tissues.
4. Bicinchoninic Acid (BCA) Protein assay kit.

### 2.2. F-TRAP Assay

1. Fluorescent TRAP reaction mix: 1.5 mM  $\text{MgCl}_2$ , 20 mM Tris-HCl, pH 8.3, 68 mM KCl, 1 mM EGTA, 0.05% Tween-20 (v/v), 50  $\mu\text{M}$  dNTPs, 2 mM spermidine, 2 U of Amplitaq DNA polymerase, 2 ng/ $\mu\text{L}$  F-TS forward primer, 2 ng/ $\mu\text{L}$  CX reverse primer. Several of the reagents are combined together in a 10 $\times$  assay buffer format for rapid setup (items 2 and 3).
2. 10 $\times$  assay buffer: 15 mM  $\text{MgCl}_2$ , 200 mM Tris-HCl, pH 8.3, 680 mM KCl, 10 mM EGTA, 0.5% Tween-20 (v/v). Store frozen in 500  $\mu\text{L}$  aliquots.

3. Oligonucleotide primers: Fluorescein-labeled TS (5'-FAATCC-GTCGAGCAGAGTT-3') and regular CX (5'-CCCTTACCCTT-ACCCTTACCCTAA-3') at 50 ng/ $\mu$ L. Make the stock primers 1000 ng/ $\mu$ L and dilute 1/20 for 50 ng/ $\mu$ L aliquots. Fluorescein-labeled primers can be used for up to 6 mo.

### **2.3. Analysis of TRAP Products**

1. ReadyMix Gel ALF grade (acrylamide). Keep refrigerated until use.
2. 10 $\times$  TBE stock buffer: 1M Tris base, 0.83M boric acid, 10 mM EDTA, pH 8.0. Must be filter sterilized through a 0.45  $\mu$ m filter for use with ALF sequencer.
3. Loading buffer: 100% deionized formamide and 5 mg/mL Blue dextran 2000. Store frozen in small aliquots.

### **2.4. Individual Reagents and Equipment**

1. Aerosol-resistant pipet tips (cat. no. P-2139, P-2149, P-2065, P-2069, P-2079, Intermountain Scientific Corporation, Kaysville, UT)
2. BCA protein assay kit, (cat. no. 23225, Pierce Chemical Company, Rockford, IL)
3. Blue dextran 2000, (cat. no. 17-0360-01, Pharmacia Biotech. Inc., Piscataway, NJ)
4. GeneAmp PCR system 9600 (Perkin-Elmer Corporation, Foster City, CA)
5. Kontes homogenization tubes with matching pestles (cat. no. 749520-0000, VWR Scientific, Seattle, WA)
6. Microcapillary 0.2 mm flat pipet tips for DNA sequencing (cat. no. 53503-012, Scientific Supply and Equipment, Seattle, WA)

## **3. Methods**

### **3.1. Preparation of Sample Extracts for F-TRAP**

#### **3.1.1. Extracts from Cell Culture (see *Notes 1–4*)**

1. Trypsinize cells from culture flask for removal, and wash once at 1500g (700 rpm) for 5 min in cold PBS or sterile culture media.

2. Count cells using a counting chamber (hemacytometer) and calculate the volume needed for 100,000 cells.
3. Pellet the cells at 1500g for 2–3 min, remove supernatant, and resuspend in 40  $\mu$ L of ice-cold protein lysis buffer.
4. Incubate 30 min on ice and centrifuge for 30 min 16,000g, 4°C.
5. Remove the supernatant to a fresh tube and store at –80°C until further use.

### 3.1.2. Patient Samples—Urine, Washing, Brushing, Fine-Needle Aspirate (FNAs) (see **Notes 5–7**)

1. Pellet the cells at 1500g for 2–3 min, remove supernatant, and resuspend in 50  $\mu$ L of ice-cold protein lysis buffer.
2. Incubate 30 min on ice and centrifuge for 30 min at 16,000g and 4°C.
3. Remove the supernatant to a fresh tube and store at –80°C until further use.

### 3.1.3. Extracts from Patient Biopsies

1. Flash freeze tissue specimens in liquid nitrogen for processing and/or future storage at –80°C.
2. With the frozen tissue, cut off flakes for homogenization using sterile blades.
3. Transfer tissue flakes to homogenization microcentrifuge tubes with matching pellet/pestle and add 200  $\mu$ L of ice-cold lysis buffer. Homogenize mixture using a drill at 750g until cells are completely dispersed.
4. Incubate on ice for 30 min and centrifuge for 30 min 16,000g and 4°C.
5. Remove the supernatant to a fresh tube and store at –80°C until further use.

### 3.1.4. Standardization of Samples for Assay

1. Determine the protein concentration, using a standard protein assay kit. The BCA protein assay kit in the microarray format was used in this study. Bovine serum albumin (BSA) in a range of dilutions is used as the protein assay standard.
2. Dilute total protein extracts to 1.5  $\mu$ g/ $\mu$ L in protein lysis buffer and store at –80°C until assayed.

**Table 1**  
**Master Mix Setup for 10 Samples**

H <sub>2</sub> O	328 $\mu$ L
10 $\times$ assay buffer	46 $\mu$ L
dNTP's	40 $\mu$ L
Spermidine (0.5M)	2 $\mu$ L
Amplitaq DNA polymerase (5 U/ $\mu$ L)	4 $\mu$ L
F-TS forward primer (50 ng/ $\mu$ L)	20 $\mu$ L
CX reverse primer (50 ng/ $\mu$ L)	20 $\mu$ L
Protein extract (1.5 $\mu$ g/ $\mu$ L) or 10,000 cell equivalent	40 $\mu$ L at 4 $\mu$ L/sample
Total	500 $\mu$ L at 50 $\mu$ L/sample

### 3.2. F-TRAP Assay (see Notes 8–10)

#### 3.2.1. F-TRAP Setup

1. Treat all TRAP reagents (excluding primers and samples) and PCR consumables under ultraviolet (UV) for at least 30 min in a closed environment to reduce PCR contamination.
2. Set up a master mix (n + 2) of the TRAP reaction buffer in a 50- $\mu$ L reaction. Keep all reagents on ice during UV treatment and master mix setup. Master mix setup for 10 total samples is shown in Table 1.
3. Set up samples in the following order to reduce contamination: negative control, protein extracts, positive control. RNase treat positive control in 1  $\mu$ g/mL RNase for 1 h at 37°C or use lysis buffer for the negative control. Several well-characterized cell lines that are known to possess telomerase activity can be used as a positive control, including HL-60, HeLa, human embryonic kidney cells 293, and MCF-7.

#### 3.2.2. TRAP Assay Conditions

1. Incubate completed reaction mixtures at room temperature for 30 min. In this phase of the assay, the telomerase in the samples will be adding 6-bp repeats to the F-TS primer.
2. The second phase is amplification of the telomerase-generated fragments. Cycling conditions are as follows: 94°C for 5 min for heat inactivation of telomerase and denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s and template extension at 72°C for 45 s for

30 cycles with a final 72°C extension of 3 min before cooling to 4°C on a hold cycle.

3. Following removal of F-TRAP products from the thermal cycler, active enzymes in the TRAP PCR products are inactivated by the addition of 1 µL of 0.1M EDTA.

### **3.3. Analysis of Products (see Note 11–16)**

#### **3.3.1. Sequencing Gel Preparation**

1. Use glass plates specific for the ALF DNA sequencer (notched plate and thermo plate) and assemble with 0.5-mm spacers according to the manufacturer's instructions.
2. Pour a 6% acrylamide gel using ReadyMix gel ALF grade (Pharmacia).
3. Allow the gel to polymerize at least 2 h before removing comb and installing to gel apparatus of the DNA sequencer. Be sure to clean off any residual acrylamide from the plate, especially from the light coupler.
4. Add filter sterilized running buffer (1X TBE) to gel apparatus of the DNA sequencer. Warm up thermo plate to achieve constant temperature before running.
5. Adjust laser beam to highest intensity without diffraction of the beam.

#### **3.3.2. Sample Loading and Gel Run**

1. Mix 5 µL of TRAP PCR product with 5 µL of gel-loading buffer.
2. Heat each sample at 95°C for 2–3 min for denaturation and quickly place on ice before loading.
3. Load all 10 µL of sample into separate wells using flat-tipped microcapillary pipet tips designed for loading sequencing gels.
4. Run the DNA sequencer at 1500 V, 38 mA, and 34 W at a constant temperature of 45°C.

#### **3.3.3. Analysis of Telomeric Repeats**

1. Observe computer screen to view real-time images of peaks representative of telomeric repeats. The F-TS and CX primers have a 2-base complementarity that will produce a primer–dimer band of approx 40 bp followed by telomeric repeats. Repeats usually appear after

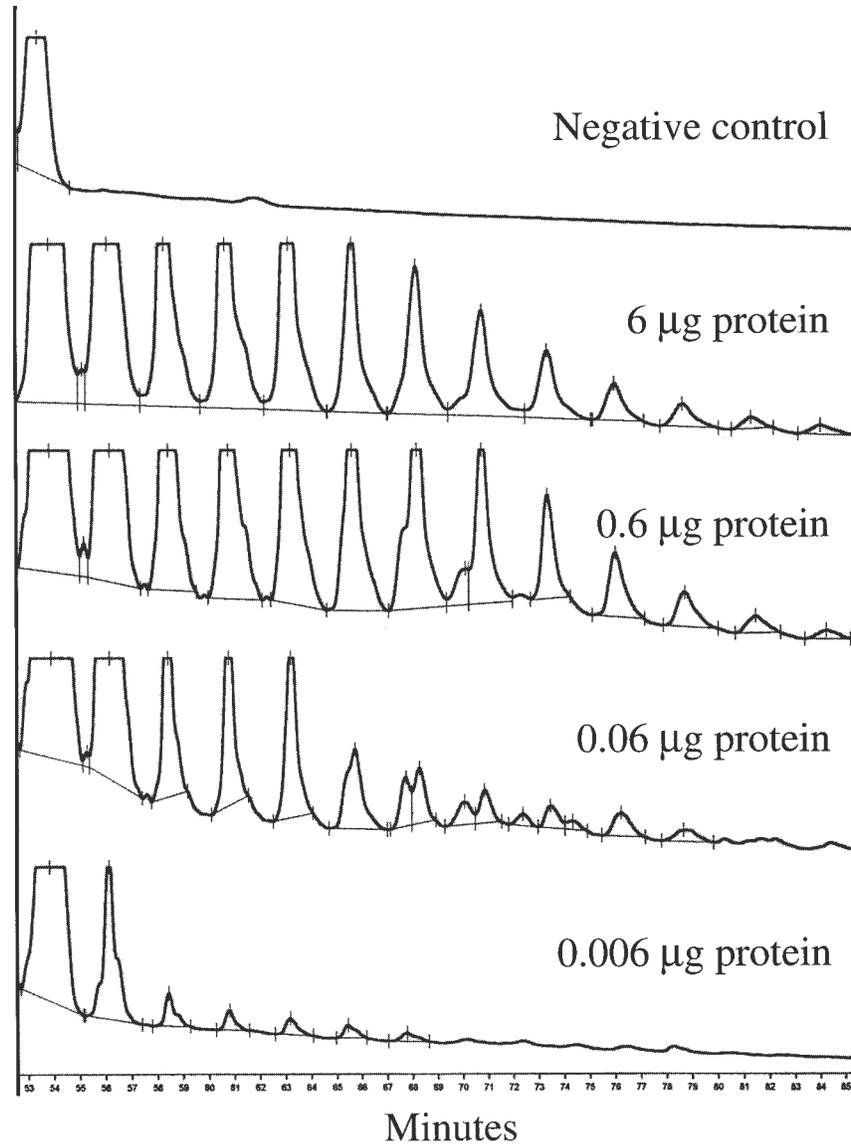


Fig. 1. Representative chromatograms demonstrating telomerase activity. The first peak represents the primer-dimer as seen in the negative control as well as the following diluted samples from a prostate tumor. The peaks exhibit a ladder effect indicative of telomeric repeats. Peak areas can then be summed using the Fragment Manager for semiquantitative measurements of telomerase activity.

13. Wash plates extremely well to remove all detergents and/or contaminants. There will be gel-to-gel inconsistencies unless the sequencing gel is prepared in a meticulous manner.
14. Clean the light coupler carefully and make sure there are no nicks or chips that could cause diffraction of the laser beam and lower the sensitivity of detection.
15. After the laser has been turned on, adjust the intensity of the beam to its brightest point and then adjust the height of the plates so that the laser intersects the individual photodiodes in a straight line. Any contaminant in the gel or mark on the plate which covers up an individual photodiode would eliminate the use of that lane by interfering with detection of a fluorescent signal.
16. Occasionally samples will have inhibitors for the thermostable *Taq* DNA polymerase; removed after subsequent dilution of samples.

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## **Nonradioactive Detection of Telomerase Activity Using a PCR–ELISA-Based Telomeric Repeat Amplification Protocol**

**Thomas Emrich and Gerlinde Karl**

### **1. Introduction**

Numerous studies on telomerase expression have consistently demonstrated the presence of telomerase activity in the vast majority of different types of cancer, as well as immortalized cells, but have failed to detect telomerase in most normal tissues (*1*). Examples include breast cancer, neuroblastoma, and cervical cancers, in which a correlation between telomerase expression and staging or clinical outcome has been demonstrated (*2*). Therefore, its very restricted, tumor-specific expression pattern makes telomerase an attractive target for diagnostic and prognostic purposes and for therapeutic intervention in a variety of human cancers

The conventional primer-extension-based assay for the detection of telomerase activity allows detection of telomerase with only limited sensitivity (*3*). This disadvantage has been overcome by the telomeric repeat amplification protocol (TRAP), in which the telomerase-mediated elongation products are amplified by PCR (*1*).

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However, because of the repetitive structure of the PCR products, unspecific amplification products resulting from staggered annealing of the reverse primer have been described (4). Additionally, another weakness in the original protocol is that telomerase-negative samples cannot be differentiated from telomerase-positive samples containing inhibitors of PCR amplification, thus giving false-negative results when analyzed for telomerase activity (5). Despite several improvements that resolve these difficulties, this method still involves the analysis of radioactively labeled reaction products by polyacrylamide gel electrophoresis (PAGE) and visualization of the gels, which is both hazardous and time-consuming, and the procedure limits the number of samples to be examined simultaneously (4,6).

Here, we describe a convenient, nonradioactive method for the detection of telomerase activity that omits the gel separation step originally described for the TRAP method. Using a simple enzyme-linked immunosorbent assay (ELISA) protocol to detect the amplification products, the method presented here is useful for sensitive detection of telomerase activity, especially when large numbers of samples have to be analyzed simultaneously. With the method described it is possible to assay a large number of samples within 5–6 h.

The specificity and sensitivity of the TRAP method and ELISA detection was examined by extracts of an immortal cell line (HEK293) and extracts prepared from a bladder carcinoma. The same extracts were analyzed in parallel with the conventional TRAP assay using a  $^{32}\text{P}$ -end-labeled TS primer. As shown in **Figure 1**, after ELISA detection strong signals were detected in samples prepared from HEK293 cells and tumor material, whereas in samples without extracts (data not shown), RNase- or heat-treated extracts, and extracts from nonmalignant cells, only background activity could be detected (**Fig. 1A**, *a* and *c*). The presence of signals corresponds to the presence of the DNA ladders typically observed for telomerase after detection by the conventional TRAP method (**Fig. 1B**, *a* and *c*). Analysis of serially diluted extracts from HEK293 cells shows that compared to

the conventional TRAP assay, as few as 10 cells could be detected clearly after ELISA detection (**Fig. 1A, b**). Similarly, clearly positive signals are obtained when analyzing about 30 ng of total protein of tumor material (**Fig. 1A, d**). Furthermore, the data demonstrate that the signals generated are linear with the number of cells and the amount of total protein in the samples (**Fig. 1A, b and d, Fig. 1B, b and d**).

The use of a hybridization probe under stringent conditions ensures that the observed signals are attributable to amplified telomerase products and allows the gel separation step in the conventional protocol to be omitted. This was verified with a large number of extracts derived from different cell lines and tissues that were analyzed with the ELISA protocol and the conventional TRAP method (data kindly provided by M. Müller, Universitätsklinikum Benjamin Franklin, Berlin, and A.-K. Bosserhoff, University of Regensburg, Regensburg, Germany). The analysis shows that equivalent results were obtained with both methods (**Fig. 2**). Compared to the conventional TRAP assay, no time-consuming and nonautomatable gel preparation and electrophoretic separation steps are required. As nonradioactive labels are used for the detection, repeated analysis of the samples without loss of activity is possible. In addition, the biotin-labeled amplification products can be separated by gel electrophoresis, blotted, and detected with an anti-biotin-enzyme conjugate. This results in the 6-nucleotide (nt) incremented DNA ladder typically observed for telomerase.

As shown above, the sensitivity of the assay is equal to or even superior to that of the conventional TRAP assay. The specificity observed is the same as with the conventional assay and has proven to be highly reproducible. Inclusion of an internal amplification standard (IS or ITAS) and normalization of the signals of the samples to that of the internal standard compensates for variations in the amplification process and allows semiquantitation of telomerase activity (*see Subheading 3.4.*). Additionally, false-negative tumor samples containing inhibitors of the amplification will be identified.

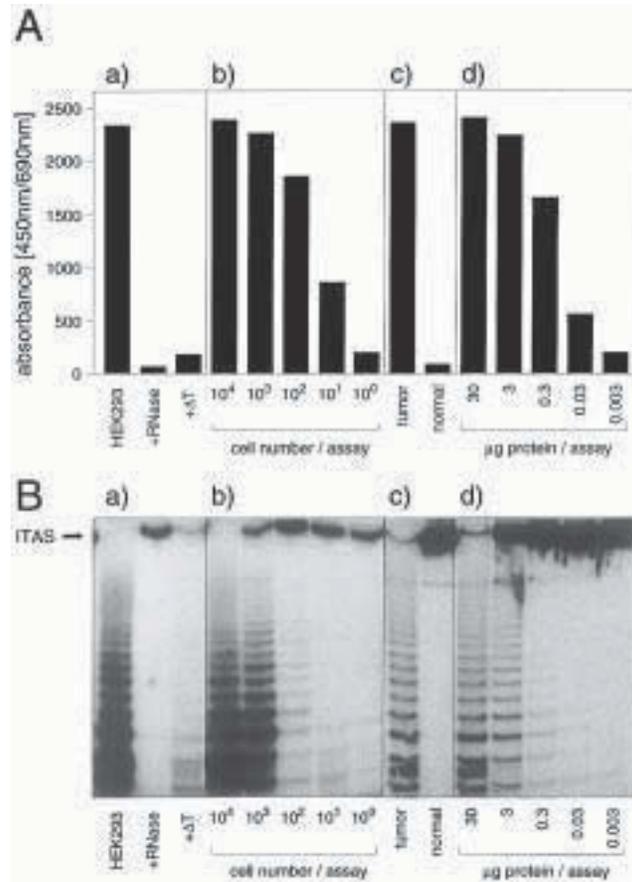


Fig. 1. Specificity and sensitivity of ELISA detection. Extracts of HEK293 cells (*a,b*), of a bladder carcinoma (*c,d*) and of normal bladder tissue (*c*) were analyzed for telomerase activity as described either by the ELISA protocol (**A**) and by the conventional TRAP assay (**B**; 25  $\mu$ L of the reaction mixture was analyzed by 10% native PAGE, and the resulting gel was exposed for 15 h to X-ray film). Extracts were assayed in the presence of a 216-bp internal standard (ITAS; 20 attg/reaction) added to the PCR mix (**B**). In *a* and *c* 10<sup>4</sup> HEK293 cells and 30  $\mu$ g of protein, respectively, were analyzed. Controls in *a* represent extracts corresponding to 10<sup>4</sup> HEK293 cells that were preincubated for 10 min at 37°C with 1  $\mu$ g of RNase (+RNase) and for 10 min at 65°C (+ $\Delta$ T), respectively, before assaying. Data are representative of at least two independent experiments. For *b* and *d*, extracts were serially 10-fold diluted in lysis buffer before adding to the reaction mixture.

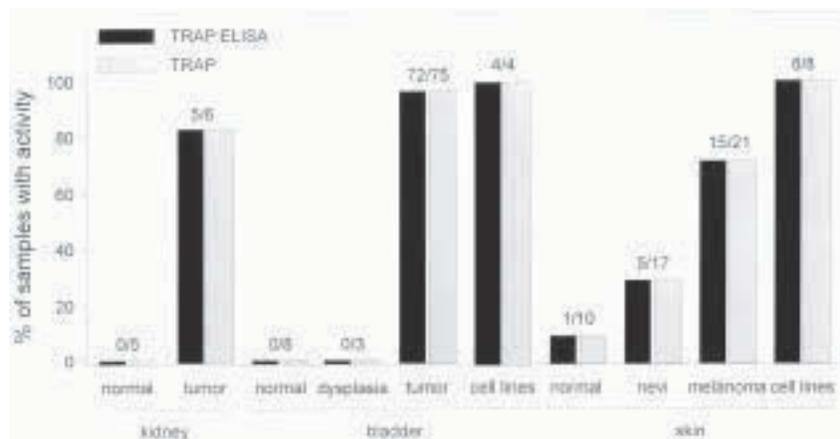


Fig. 2. Detection of telomerase activity in tumor and normal tissues obtained from kidney, bladder, and skin biopsy and in immortal bladder and different other cell lines (bladder: F975, J82, ScaBER, UM-UC-3; skin: Mel Im, Mel Ho, Mel Wei, Mel Ei, Mel Ju, Mel Juso, I-ITZ-19, SKMel 28). Total protein (10  $\mu$ g) was assayed for telomerase activity by both ELISA detection and the conventional TRAP method. The numbers above the columns represent the portion of samples with telomerase activity relative to the total number of samples analyzed.

## 2. Materials

Unless stated otherwise, all reagents were purchased from Roche Molecular Biochemicals and are of the highest grade possible.

Determining telomerase activity requires both the addition of telomeric repeats to a primer by the activity of telomerase contained in the sample and their subsequent amplification by PCR. Therefore, there is a need for extreme caution to prevent RNase/DNase contamination that might cause degradation of the internal, telomerase-associated RNA template, as well as polymerase chain reaction (PCR) carryover contamination resulting in false-positive signals. To allow achievement of reliable results, the entire assay procedure must be performed under nuclease-free conditions. Only nuclease-free solutions (e.g., diethyl pyrocarbonate [DEPC] treated) are recommended, and they should be stored in appropriate aliquots, separate from other reagents in the laboratory. To minimize the risk

of carryover contamination, the workplaces for sample preparation, TRAP reaction, and detection of amplicons to physically separate.

1. *TeloTAGGG* Telomerase PCR ELISA contains all of the reagents for performing the complete assay procedure except the internal standard and standard-specific hybridization buffer.
2. *TeloTAGGG* Telomerase PCR ELISA<sup>PLUS</sup> contains all of the reagents for performing the complete assay procedure
3. PBS: 137 mM NaCl, 2.7 mM KCl, 10.2 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>.
4. Lysis buffer: 10 mM Tris-HCl (pH 7.5), 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.5% CHAPS, 5 mM β-mercaptoethanol, 0.1% AEBSF (Pefabloc SC, 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride), 10% glycerol. Store at –20 °C.
5. Reaction buffer, 2×: 40 mM Tris-HCl (pH 8.3), 126 mM KCl, 3 mM MgCl<sub>2</sub>, 2 mM EGTA, 0.1% Tween-20, 50 μM each deoxynucleoside triphosphate, 0.2 μg of PI-TS primer (5'-Biotin-AATCCGTCG-AGCAGAGTT-3'), 0.2 μg of P2 anchor primer, 4 U of *Taq* DNA polymerase. Store at –20 °C.
6. Internal standard: 0.001 attomole/μL. The 216-bp internal standard contains the sequence (4620–4779 nt) from the bacterial CAT gene flanked by the PI-TS primer and P2 anchor primer (acct. no. EB\_SYN:AGPSV2CAT; Roche) (see **Note 1**). Store at –20°C.
7. Control template: 5'-AATCCGTCGAGCAGAG(TTAGGG)<sub>7</sub>TTAG-3'; 0.001 attomole/μL. The control template used is identical to a telomerase elongation product with 8 telomeric repeats. Store at –20°C.
8. Denaturation solution: 0.2M NaOH. Store at room temperature.
9. Hybridization buffer T: 5'-digoxigenin (DIG)-labeled oligonucleotide complementary to telomeric repeats in hybridization buffer. Store at –20°C.
10. Hybridization buffer IS: 5'-DIG-labeled oligonucleotide complementary to the internal standard in hybridization buffer. Store at –20°C
11. Anti-DIG-HRP (polyclonal anti-DIG antibody from sheep, conjugated to horseradish peroxidase): 10 mM in conjugate buffer. Prepare immediately before use; do not store.
12. Streptavidin-coated microtiter plate (MTP), precoated with streptavidine and postcoated with blocking reagent. Store at 4°C.

13. Washing buffer: 1.5 mM Na citrate (pH 7.0), 150 mM NaCl. Store at 4°C.
14. Substrate solution: 0.1 mg/mL TMB (3,3',5,5'-tetramethylbenzidine) in 2 mM citrate acid, 100 mM Na acetate, 0.1 % H<sub>2</sub>O<sub>2</sub>. Prepare immediately before use; do not store.
15. Stop reagent: 2M H<sub>2</sub>SO<sub>4</sub>. Store at room temperature.
16. RNase: RNase, DNase-free, 500 ng/μL in 10 mM Tris-HCl (pH 7.0), 5 mM CaCl<sub>2</sub>, 50% glycerol (v/v). Store at -20°C.

### 3. Methods

#### 3.1. Preparation of Cell Extracts from Cells

1. Harvest and count cells using standard methods (e.g., trypan blue staining, using a hemocytometer, e.g., Neubauer chamber).
2. Transfer  $2 \times 10^5$  cells per single reaction into a fresh Eppendorf tube.
3. Pellet cells at 3000g for 5 min in a refrigerated centrifuge at 4°C.
4. Carefully remove the supernatant, resuspend the cells in PBS, and repeat the centrifugation step. If the TRAP reaction and ELISA detection are not performed immediately after the extract preparation, the pelleted cells can be stored at -80°C or less until use.
5. Resuspend the pelleted cells in 200 μL of lysis buffer, precooled on ice by retropipeting at least three times.
6. Incubate on ice for 30 min.
7. Centrifuge the extract at 16,000g for 20 min at 4°C.
8. Carefully remove the supernatant and transfer to a fresh tube. To ensure that no cellular debris of the pelleted cells is transferred, pipeting of only 175 μL of the cell extract is recommended.
9. When not performing the TRAP reaction, immediately shock freeze the cell extract in aliquots in liquid nitrogen and store the extracts at -80 °C.

#### 3.2. Preparation of Cell Extracts from Frozen Tissues

1. Prepare cryostat sections of 10 to 15-μm thickness.
2. Transfer approx 50 sections into a sterile reaction tube containing 200 μL of ice-cold lysis buffer and resuspend on ice by retropipeting at least three times (*see Note 2*).

3. Incubate on ice for 30 min.
4. Carefully remove the supernatant and transfer to a fresh tube. To ensure that no cellular debris of the pelleted cells is transferred, pipeting of only 175  $\mu\text{L}$  of the cell extract is recommended.
5. When not performing the TRAP reaction, immediately shock freeze the cell extract in aliquots in liquid nitrogen and store the extracts at  $-80^{\circ}\text{C}$ .

### **3.3. PCR Amplification (TRAP Reaction)**

All pipeting steps should be done on ice.

1. For each sample to be tested and the control template, transfer 25  $\mu\text{L}$  of reaction buffer and 5  $\mu\text{L}$  of IS into a tube suitable for PCR amplification (*see Note 3*).
2. Transfer 30  $\mu\text{L}$  of the reaction mixture/master mix into tubes suitable for PCR amplification per PCR reaction.
3. Samples: Add 1–3  $\mu\text{L}$  of cell extract (corresponding to  $10^3$ – $3 \times 10^3$  cell equivalents or 0.5–10  $\mu\text{g}$  of total protein) per tube (*see Note 4*).
4. Negative controls (*see Note 5*): Add 1–3  $\mu\text{L}$  of the corresponding heat-treated cell extract (corresponding to  $10^3$ – $3 \times 10^3$  cell equivalents or 0.5–10  $\mu\text{g}$  of total protein) per tube .
5. Control template (*see Note 6*): Pipet 1  $\mu\text{L}$  of the control and 1  $\mu\text{L}$  of lysis buffer into two separate tubes.
6. Add nuclease-free, double distilled water to a final volume of 50  $\mu\text{L}$ .
7. Transfer the tubes to a thermal cycler and perform a combined primer elongation/amplification reaction by the following protocol (*see Note 7*):  $25^{\circ}\text{C}$  for 10 min and  $94^{\circ}\text{C}$  for 5 min for one cycle each, and then at  $94^{\circ}\text{C}$  for 30 s,  $50^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 90 s for 30 cycles.

### **3.4. Hybridization and ELISA Detection**

1. Per sample, pipet 10  $\mu\text{L}$  of denaturation solution into two reaction tubes each (see pipeting scheme, **Fig. 3A**). For large numbers of samples, use of nuclease-free uncoated microtiter plates is recommended.
2. Per vial, add 2.5  $\mu\text{L}$  of the amplification product and incubate at room temperature for 10 min.

3. Add 100  $\mu\text{L}$  of hybridization buffer T to one vial and 100  $\mu\text{L}$  of hybridization buffer IS to the other vial, and mix thoroughly by vortexing briefly.
4. According to the pipeting scheme, transfer 100  $\mu\text{L}$  of each mixture per well of a streptavidin-coated microtiter plate and cover the wells with the self-adhesive cover foil.
5. Incubate the microtiter plate at 37°C on a shaker (300 rpm) for 2 h.
6. Remove the hybridization solutions completely.
7. Wash each well three times with 250  $\mu\text{L}$  of washing buffer for a minimum of 30 s each and remove washing buffer carefully.
8. Add 100  $\mu\text{L}$  of anti-DIG-HRP per well, cover the microtiter plate with cover foil and incubate at room temperature (18–22°C) for 30 min while shaking at 300 rpm.
9. Remove the solution completely. Rinse five times with 250  $\mu\text{L}$  of washing buffer per well for a minimum of 30 s each, and remove washing buffer carefully.
10. Per well, add 100  $\mu\text{L}$  of TMB substrate solution prewarmed to room temperature, cover the wells with cover foil, and incubate for color development at room temperature (18–22°C) for 10–20 min while shaking at 300 rpm.
11. Without removing the reacted substrate, add 100  $\mu\text{L}$  of stop reagent per well to stop color development (*see Note 8*).
12. Using a microtiter plate (ELISA) reader, measure the absorbance of the samples at 450 nm (with a reference wavelength of approx 690 nm) within 15 min after addition of the stop reagent.

### **3.5. Quantification of Telomerase Activity**

The level of telomerase activity in a given sample is determined by comparing the signal from the sample to the signal obtained using a known amount of a control template (TS8). The control template used is identical to a telomerase elongation product with 8 telomeric repeats. To include variances of the amplification process and to control the amplification inhibition by potential inhibitors included in the sample material, both signals are normalized to the signal of an internal amplification control (IS or ITAS; *see Note 1*). RTA units within different samples in an experiment are obtained using the formula in **Figure 3B**.

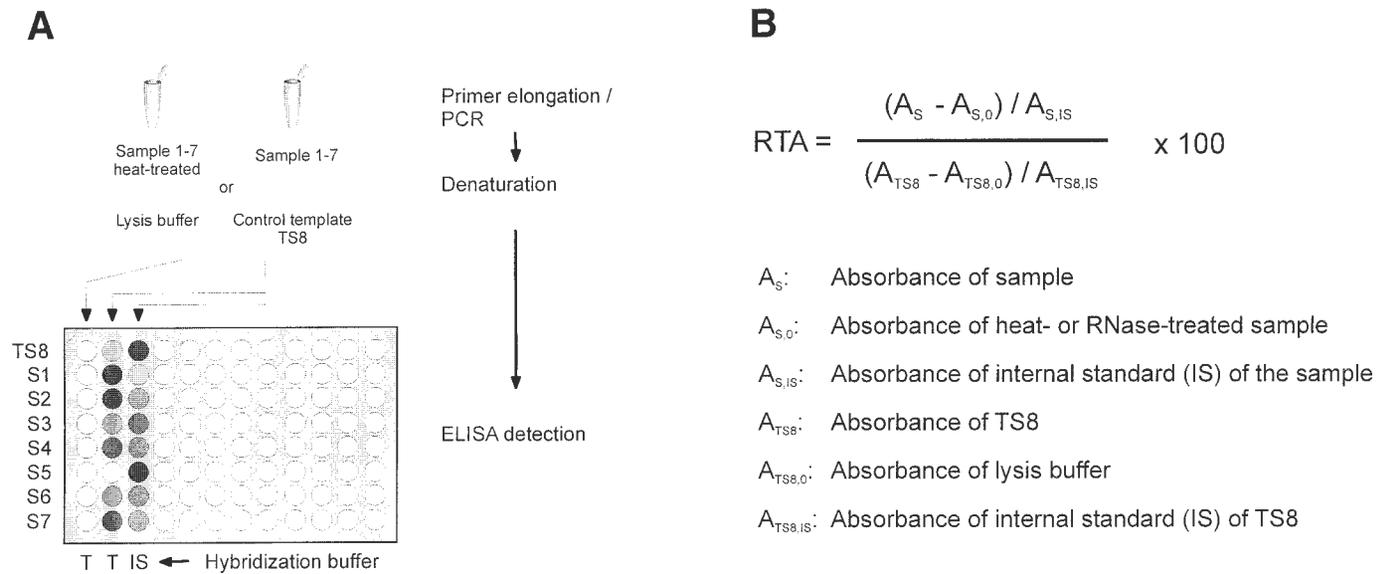


Fig. 3. (A) Pipeting scheme for the TRAP method and ELISA detection using an internal amplification standard (IS or ITAS). (B) Formula used for the quantitation of telomerase activity defined as telomerase activity (RTA) units.

#### 4. Notes

1. Several ISs have been reported in the literature (4,6), of which a heterologous 36-bp IS is most often used (4). However, the use of the 36-bp IS has been shown to carry the risk of producing false-negative results: Because of its short length, it can be amplified in samples that clearly contain *Taq* DNA polymerase inhibitors (5). To overcome this problem the IS used here is a 216-bp homolog standard that allows clear detection of *Taq* DNA polymerase inhibitors. Additionally, it produces a fragment that is long enough not to interfere with the visualization of the telomerase ladder when the products are analyzed after electrophoretic separation.
2. Alternatively, if a microtome is not available, thin slices of frozen tissue specimens may be prepared on sterile disposable Petri dishes with surgical disposable knife blades to obtain thin flakes, which are then immediately transferred to homogenization tubes containing 200  $\mu$ L of ice-cold lysis buffer. Homogenize on ice with a motorized pestle until consistency is uniform.
3. To ensure the same reaction conditions for all samples, prepare a master mix to analyze samples and control templates simultaneously; e.g., if 7 samples including negative controls are to be analyzed, mix 425  $\mu$ L of reaction mixture and 85  $\mu$ L IS.
4. To obtain valid quantitative analysis each sample should contain the same amount of cell equivalents or have the same protein content.
5. As telomerase essentially requires integrity of its internal RNA component as a template, preincubation of the cell or tissue extract with RNase (DNase-free) will fully destroy telomerase activity contained in the extract and offer convenient specificity control. For this control, incubate 5  $\mu$ L of cell extract typically corresponding to 5000 cell equivalents, or 50  $\mu$ g of protein if extract from tissue samples is used, with DNase-free RNase at a concentration of 1  $\mu$ g/ $\mu$ L for 20 min at 37°C. An aliquot of 1–2  $\mu$ L of the RNase-treated extract is used in the assay.
6. The level of telomerase activity in a given sample is determined by comparing the signal from the sample to the signals obtained using known amounts of a control template (TS8) that is identical to a telomerase elongation product with 8 telomeric repeats.
7. The program mentioned has been established for use with the GeneAmp PCR System 9600 and 9700 Thermal Cycler (Perkin

Elmer). It has been shown in a number of experiments that overlaying mineral oil on top of the reaction mixture for the telomerase-mediated primer elongation does not influence the results. However, depending on the performance characteristics of other thermal cyclers, minor modifications of the protocol and overlaying the reaction mixture with mineral oil to prevent water condensation might be required.

8. Addition of the stop reagent causes the reacted peroxidase substrate to change in color from blue to yellow and is required to achieve maximal sensitivity.

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## ***In Situ* TRAP Assay Detection of Telomerase Activity in Cytological Preparations**

**Kazuma Ohyashiki and Junko H. Ohyashiki**

### **1. Introduction**

In 1994, a highly sensitive assay to detect telomerase activity, called a telomeric repeat amplification protocol (TRAP) assay, was reported by Kim et al. (1) with the precise method described in 1995 by Piatyszeck et al (2). Many modifications of the original TRAP assay were made later. One example is the use of an internal telomerase assay standard (ITAS) to semiquantify the level of telomerase activity (3). Another approach to semiquantify the telomerase activity, using a stretch PCR assay, was reported by Tatematsu et al. With this method, it is possible to detect telomerase activity at the single cell level (4). More recently, a commercially available kit has been provided (5). Enabling the detection of telomerase activity easily. A body of clinical information has been accumulating and its implications are being obtained (6,7).

These TRAP assays rely on the use of telomerase extracted from homogenized tissues, and as a result, it is difficult to determine which cells within a culture express the observed telomerase activity.

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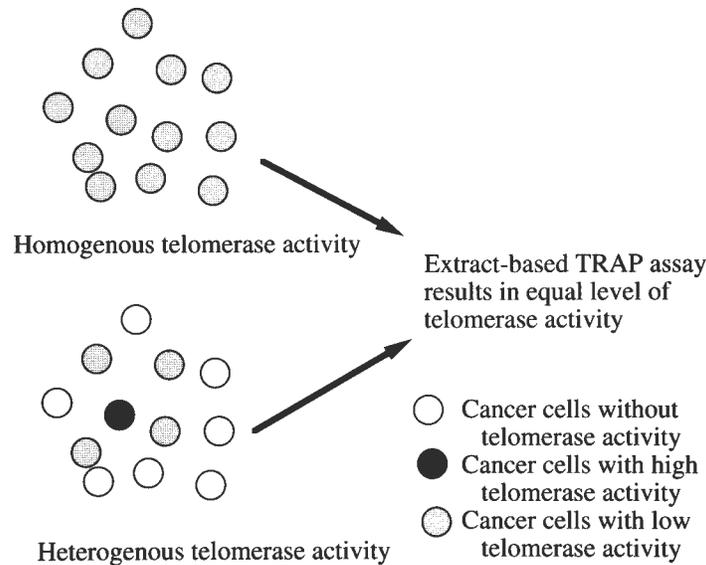


Fig. 1. Schematic presentation of telomerase activity at the cellular level. Using the original extract-based TRAP assay, it is difficult to determine whether all of the cells express an equal or heterogenous level of telomerase activity.

It has been suggested that tumor tissues contain a heterogenous pattern of telomerase activity (**Fig. 1**). One approach to resolve this problem by the use of *in situ* hybridization of the catalytic subunit of telomerase, the expression of which has been found to be closely related to telomerase activity. To answer these problems we have developed an *in situ* telomerase assay using an *in situ* PCR technique to detect telomerase activity directly (**8**).

## 2. Materials

### 2.1. Cell Preparation

1. Hypotonic solution: 1.2114 g Tris-HCl, 0.7456 g KCl, and 0.2033 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  made up to 1 L with distilled water.
2. Hypertonic solution: 16 g NaCl, 0.4 g KCl, 5.795 g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , and 0.4 g  $\text{KH}_2\text{PO}_4$  made up to 1L with distilled water.

## 2.2. In Situ TRAP Assay

1. Reaction mixture: 20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 63 mM KCl, 0.05% Tween-20, 1 mM EGTA, 50 mM deoxynucleoside triphosphates, 1 mg of T4 gene 32 protein (Boehringer Mannheim, Indianapolis, IN), 0.1 mg/mL of bovine serum albumin, 2 units of *Taq* DNA polymerase.
2. Oligonucleotide primers: Fluorescein-labeled TS primer (5'-AAT-CCGTCGAGCAGAGTT-3') and fluorescein-labeled CX reverse primer (5'-CCCTTACCCTTACCCTTACCCTAA-3') at 10 pmol per slide (*see Note 1*).
3. Slide mountant: 1:1 MacIlvaine buffer/glycerin solution (v/v).

## 2.3. Other Equipment

1. Hybrid OmniSlide System thermocycler (National Labnet Co., Woodbridge, NJ).
2. Fluorescence microscope (Nikon, Tokyo, Japan).

## 3. Methods (*see Fig. 2*)

1. Resuspend cells in 30 mL of filtered hypotonic solution. Mix carefully 15×. Add 10 mL of filtrated hypertonic solution immediately. Pellet cells in cooled medium or saline (*see Notes 2 and 3*).
2. Cytospun the pelleted cells (800g for 3 min) onto silane-coated nonfluorescent glass slides and air-dry rapidly using a flow of cool air (*see Note 4*).
3. Place 25 μL of TRAP reaction buffer plus FITC-labeled TS forward primer within each slide frame and incubate the slides for 30 min at 22°C in the dark.
4. Add an additional 25 μL of reaction buffer plus FITC-labeled CX reverse primer, seal the coverslips, and heat the slides to 90°C for 1.5 min to inactivate telomerase (*see Note 5*).
5. Amplify extended TS primers by using conditions of 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min.
6. Remove the slides from the coverslips, wash in tap water (*see Note 6*), and seal with a glass cover using MacIlvaine buffer/glycerin solution.
7. Examine cells and photograph with a fluorescence microscope using a B-filter (*see Notes 7 and 8, and Fig. 3*).

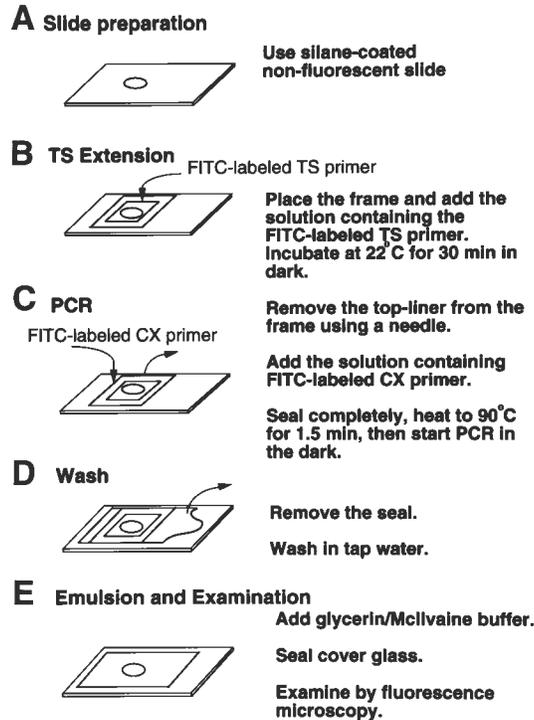


Fig. 2. Protocol of the in situ TRAP assay.

#### 4. Notes

1. Avoid multiple freeze–thawing of the FITC-TS and FITC-CX primers. Either use fresh primers for each reaction or freeze–thaw only twice.
2. Cell suspensions that have been treated with hypotonic and hypertonic solutions can be stored at  $-80^{\circ}\text{C}$  until use.
3. Positive control cells should be selected carefully, as cultures over 1 wk old have been found to have few fluorescence-positive cells. It is therefore recommended to use subconfluent cultures 1–2 d after plating.
4. After cell attachment on the slides, it is important to dry the cells quickly and completely (at  $22^{\circ}\text{C}$ ; do not use a hot air dryer) for a period not exceeding 5 min. The slides should then be stored at  $-20^{\circ}\text{C}$  until used in the procedure.
5. It is important that the PCR reaction mixture is sealed onto the slides. Excess reaction mixture is included to ensure the exclusion of air bubbles in the sealed PCR reaction mixture.

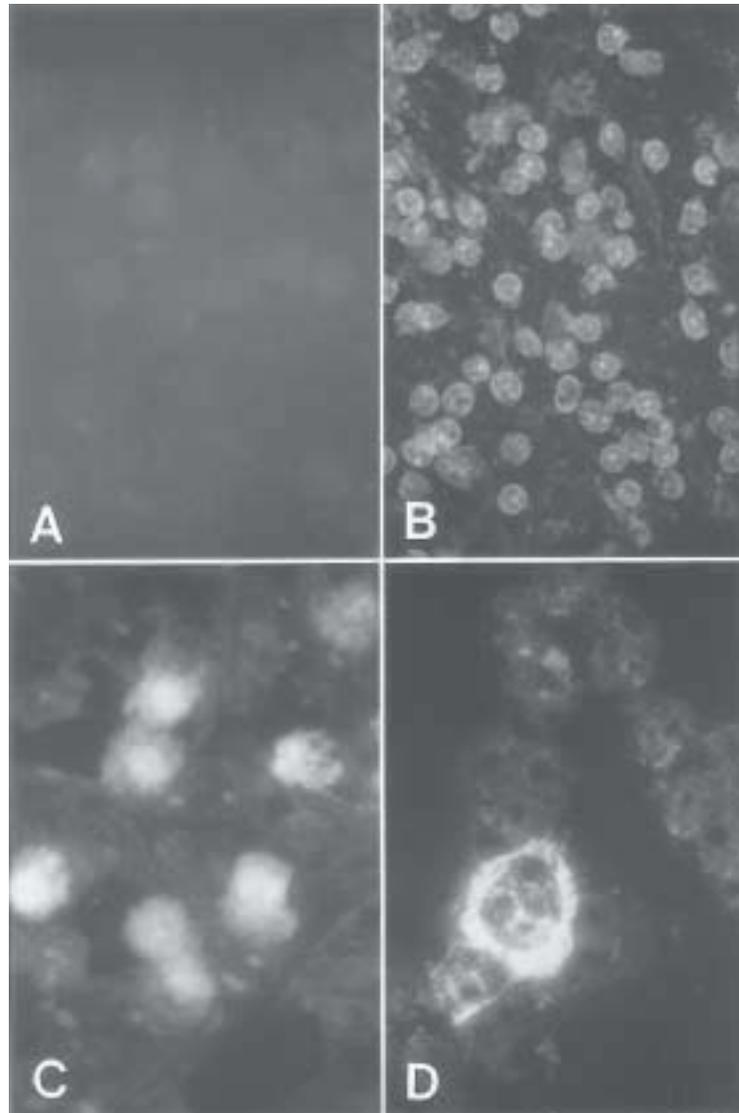


Fig. 3. Typical results of the *in situ* TRAP assay. The fluorescent signal in normal resting lymphocytes is very weak (**A**); whereas a strong nuclear signal is observed in stimulated lymphocytes with bright fluorescence in the nucleoli (**B**). In neoplastic cells, bright fluorescence is detectable in nucleoli corresponding to telomerase activity (**C**). In granulocytes, punctate bright fluorescence is noted but it is nonspecific and does not correspond to telomerase activity (**D**) (see **Note 9**).

6. Washing the slides carefully after the PCR assay to avoid losing the cells.
7. Fading of the fluorescent signal occurs rapidly under examination and care should be taken to avoid unnecessary exposure. Exposure for photography times varies from between 1 and 3 s for low-magnifications to approx 30 s for high-magnification objectives.
8. When not being examined, the slides should be stored in the dark at all times to avoid fading of the signal.
9. Nonspecific fluorescence is sometimes noted in cells such as granulocytes, but it is cytoplasmic and punctate in character and easily distinguished from positive telomerase fluorescence.

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## Biotinylated Primer for Detecting Telomerase Activity Without Amplification

Daekyu Sun

### 1. Introduction

The activation of telomerase, proposed as a critical set for cell immortalization to overcome cellular senescence, was believed to be associated with the malignant progression of human tumours (1–4). Therefore human telomerase appears to be an attractive new target for designing anti-cancer agents, as well as an important diagnostic marker of human cancer (5–7). For these reasons, it is necessary to develop telomerase assay methods that can be used for the quantitative measurement of telomerase activity.

An alternative assay method was designed to overcome the inherent difficulties of known telomerase assay methods (1,8). In the newly developed assay, 5'-biotinylated (TTAGGG)<sub>3</sub> primer is used instead of (TTAGGG)<sub>3</sub>, so that telomerase reaction products can be immobilized by the addition of Dynabeads streptavidin, which binds selectively to the 5'-biotinylated primers (9). Dynabeads streptavidin comprises uniform, supramagnetic, 2.8 μM polystyrene spheres covalently coupled with streptavidin protein. The high affinity of the streptavidin/biotin interaction ( $K_d = 10^{-15}$ ) allows for the rapid and efficient isolation of biotin-labeled target molecules (10).

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These magnetic bead–target complexes can be separated easily from the suspension by a magnet and washed extensively with washing buffers to eliminate the background from the [ $\alpha^{32}\text{P}$ ]dGTP and high molecular weight (mol wt) DNA labeled with  $^{32}\text{P}$  by contaminating DNA polymerases in the cell extracts. Finally, the telomerase reaction products can be dissociated from the magnetic beads with a protein denaturant treatment (5.0M guanidine-HCl) and subsequently analyzed using polyacrylamide gel electrophoresis. As shown in **Figure 1**, primer extension activity of human telomerase is sensitive to treatments with RNase A and proteinase K, and is increased by an increasing concentration of nucleotide substrate. In the first extension, the major band corresponds to the 5'-TTAG\* (\* indicates incorporated [ $\alpha^{32}\text{P}$ ]dGTP into telomeric primer) telomerase-extended species (22 bp) with less intense bands for the successive exonuclease-produced 5'TTA and 5'-TT species. In the following extensions, six telomeric nucleotides (5'-G\*G\*TTAG\*) were added to telomeric primers, resulting in the generation of 6-bp ladders (28, 34, 40, 46 bp, etc). With the newly developed method discussed here, it was possible to detect telomerase in HeLa extracts equivalent to several hundred cells without using PCR amplification of telomerase reaction products (9).

## 2. Materials

1. Telomerase reaction buffer: 50 mM Tris-OAc, pH 8.5, 50 mM KCl, 1mM MgCl<sub>2</sub>, 5 mM  $\beta$ -mercaptoethanol, 1 mM spermidine. Store at  $-20^{\circ}\text{C}$ .
2. Dynabeads resuspension buffer: 10 mM Tris-HCl, pH 7.5, and 2M KCl. Store at  $4^{\circ}\text{C}$ .
3. Dynabeads washing buffer: 10 mM Tris-HCl, pH 7.5 and 1M NaCl. Store at  $4^{\circ}\text{C}$ .
4. Cell lysis buffer: 10 mM Tris-HCl, pH 7.5, 1 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride, 5 mM  $\beta$ -mercaptoethanol, 1 mM dithiothreitol (DTT), 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS). Store at  $4^{\circ}\text{C}$ .
5. Phosphate buffered saline (PBS): 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM NaH<sub>2</sub>PO<sub>4</sub>, and 140 mM NaCl, pH 7.4. Store at  $4^{\circ}\text{C}$ .

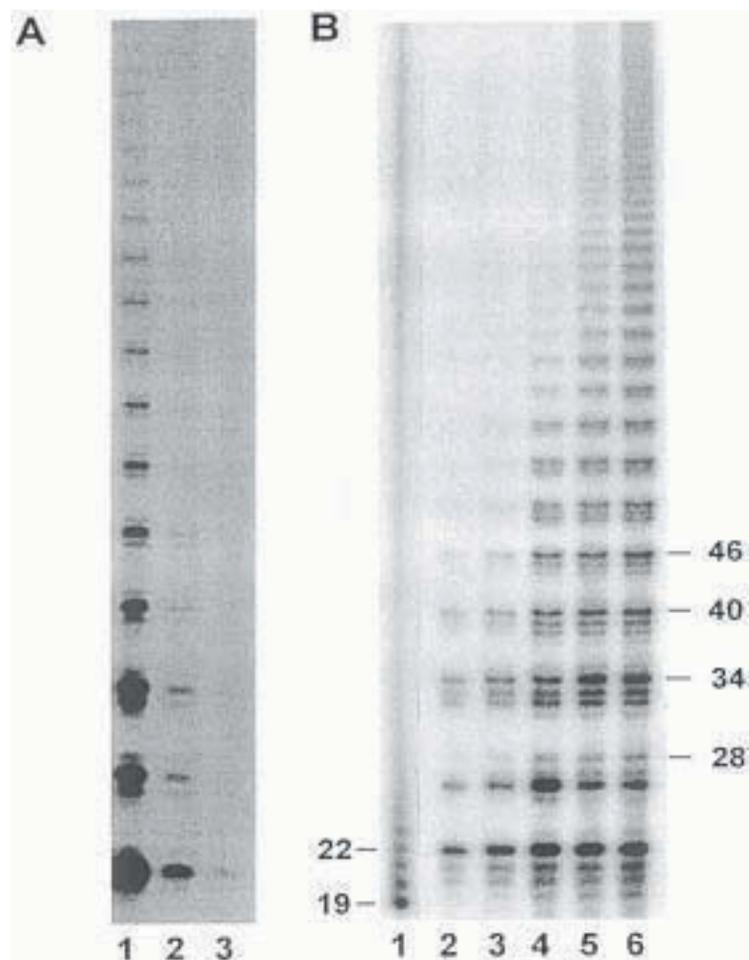


Fig. 1. (A) Telomerase assays were carried out under standard reaction conditions with 4  $\mu$ L of HeLa cell extracts. Lane 1 represents telomerase reaction products without any treatment; lanes 2 and 3 represent the telomerase reactions with proteinase K (100  $\mu$ g/mL) and RNase A (50ng), respectively. (B) Effect of dGTP concentration on the activity of human telomerase. Lane 1 shows terminal transferase reaction products of 5'-biotinylated 18-mer primer as size markers. In lanes 2–6, telomerase reactions were carried out with dGTP concentrations of 0.6, 1.2, 2.4, 4.8 and 9.6  $\mu$ M under standard reaction conditions containing 1 mM dATP and 1 mM dTTP.

6. Cell washing buffer: 10 mM HEPES-KOH, pH 7.5, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, and 1 mM DTT. Store at 4°C.
7. DNA extraction buffer: 5.0M guanidine-HCl.
8. Dynabeads streptavidin suspension (Dynabeads M-280 streptavidin, Dynal Co., Lake Success, NY, USA). Store at 4°C.
9. Magnetic rack (Dynal MPC).
10. 5'-biotinylated (TTAGGG)<sub>3</sub> primer (Genosys, The Woodlands, TX). Store at -20°C.
11. Terminal transferase enzyme and nucleotide solutions (Promega, Madison, WI). Store at -20°C.
12. [ $\alpha^{32}\text{P}$ ]dGTP (800 Ci/mmol) (DuPont-NEN, Boston, MA). Store at -20°C.
13. X-ray film (Kodak, Biomax-MS), intensifying screens for Biomax films and developing chemicals (Eastman Kodak, New Haven, CT).

### 3. Methods

#### 3.1. Preparation of Cell Lysate (S100)

1. Gently wash cultured HeLa cell suspension (National Cell Culture Institute, Minneapolis, MN) in PBS. Prepare cell pellet by centrifugation for 10 min at 2500g.
2. Resuspend cell pellet in ice-cold cell washing buffer and prepare cell pellet at 10,000g for 1 min at 4°C.
3. Add ice-cold cell lysis buffer (10<sup>6</sup> cells/20 $\mu$ L) to cell pellet and incubate for 60 min on ice (*see Note 1*).
4. Centrifuge cell lysate in an ultracentrifuge at 100,000g at 4°C for 1 h and transfer the resulting supernatant to a new tube.
5. Adjust volume of cell lysate (S100) to 20% glycerol and store in -80°C freezer.

#### 3.2. Telomerase Assay and Analysis

The assay was performed by using 5'-biotinylated (TTAGGG)<sub>3</sub> as a telomere primer instead of (TTAGGG)<sub>3</sub>, which is normally used.

1. Wash the Dynabeads streptavidin twice in the Dynabeads resuspension buffer.
2. Prepare the telomerase reaction mixture (20 mL) in an Eppendorf containing 50 mM Tris-OAc, pH 8.5, 50 mM KCl, 1 mM MgCl<sub>2</sub>,

5 mM  $\beta$ -mercaptoethanol, 1 mM spermidine, 1  $\mu$ M telomere primer, 2.4  $\mu$ M [ $\alpha^{32}$ P]-dGTP (800 Ci/mmol), 1 mM dATP, and 1 mM dTTP with 4  $\mu$ L of cell lysate.

3. Incubate at 37°C for 1 h.
4. Add 20–30 $\mu$ L of prewashed Dynabeads streptavidin suspension to the reaction mixtures to stop the reaction.
5. Incubate Dynabeads streptavidin/target complex (biotinylated primers) by vortexing for at least 30 min at room temperature to immobilize biotinylated primers to Dynabeads streptavidin (*see Notes 2–4*).
6. Add 80 $\mu$ L of Dynabeads washing buffer to Dynabeads streptavidin suspension and vortex the tubes briefly.
7. For magnetic separation, place tubes in a magnetic particle separator for 1–2 min. During this time the Dynabeads streptavidin/target complex will be attracted to the walls of the tubes by the magnetic field.
8. Remove the supernatant by aspiration with a pipette while the tube remains in the magnetic particle separator (*see Note 5*).
9. Resuspend Dynabeads Streptavidin/target complex in 120 $\mu$ L of Dynabeads washing buffer by brief vortexing.
10. Place the Eppendorf tubes in a magnetic particle separator for 1–2 min and remove the supernatant by aspiration with a pipet while the tubes remain in the magnetic separator.
11. Repeat **steps 9 and 10** for at least 5 times (*see Note 6*).
12. Add 200 $\mu$ L of DNA extraction buffer, after removing the supernatant. Dissociate the biotinylated primers from Dynabeads streptavidin by heating for at least 20 min at a minimum temperature of 90°C with occasional vortexing (*see Note 7*).
13. Place the Eppendorf tubes in the magnetic particle separator for 5–10 min and transfer the supernatant to new tubes.
14. Add 0.1 vol of 4.0M ammonium acetate and 4  $\mu$ L of yeast tRNA solution (10 mg/mL).
15. Precipitate the nucleic acid by ethanol precipitation, followed by centrifugation and drying of the pellet. Dissolve the DNA in 6 $\mu$ L of alkaline sequencing dye.
16. Electrophorese on an 8% polyacrylamide sequencing gel.
17. Dry the gel on filter paper and expose it to sensitive film (Kodak, Biomax-MS), using intensifying screens, for more than 24 h (*see Note 8*).
18. Analyze the autoradiograph by using a densitometer (ImageQuant, Molecular Dynamics).

#### 4. Notes

1. Repeatedly pass the cell suspension in lysis buffer through a pipet tip to ensure complete cell lysis.
2. To ensure that the biotinylated primers bind to Dynabeads streptavidin, continuous agitation of Dynabeads streptavidin with the telomerase reaction is recommended for at least 30 min at room temperature. If the intensity of the telomerase signal is too low, the binding time can be increased by up to 1 h to improve the binding efficiency.
3. The exact amount of Dynabeads streptavidin should be optimized by preliminary experiments.
4. The aggregation of Dynabeads streptavidin in suspension decreases the binding efficiency of target molecules to Dynabeads streptavidin. Contact the manufacturer or switch to a new brand of Dynabeads streptavidin.
5. Avoid aspirating out the bead, which could lead to a low signal.
6. If the [ $\alpha^{32}\text{P}$ ]dGTP background and nonspecific high mol wt DNA appear frequently on the autoradiograph, increase the number of washing steps or transfer Dynabeads streptavidin suspension to new tubes during the washing cycle.
7. The incubation can be increased up to 40 min, if a low signal is observed.
8. Gels can be dried on DE81 ion exchange filter paper without fixing gels to improve the intensity of signals.

#### Acknowledgment

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## Whole-Cell and Microcell Fusion for the Identification of Natural Regulators of Telomerase

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Lucie Jetté, and Mario Chevrette

### 1. Introduction

The ability of tumor cells to grow indefinitely contrasts sharply with the growth of normal cells. When normal human cells are grown *in vitro*, they undergo a limited number of divisions. This property, termed replicative senescence, is lost when cells become tumorigenic. However, whole-cell hybrids between normal and immortal cells are mortal, indicating that cellular senescence is dominant and normal cells contain genes that limit cell growth (1,2). These findings have been confirmed and expanded by microcell transfer studies in which individual chromosomes from donor cells are introduced into recipient cells. Depending on the recipient cells, transfer of at least seven different human chromosomes has been shown to induce cellular senescence (3–9). Thus, normal cells have developed many mechanisms to prevent unlimited cell growth. One of them is cellular suicide, or apoptosis, and is controlled (at least in part) by the proteins encoded by two tumor suppressor genes, namely *p53* (10)

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and *RB* (11). Another apparent mechanism is the progressive shortening of the ends of chromosomes (telomeres), following each cycle of cellular replication, leading to a state of cellular senescence after the completion of a certain number of replicative cycles (12).

In all species telomeres are composed of a series of tandemly repeated DNA sequences ( $[\text{TTAGGG}]_n$  in humans and other vertebrates) that interact with protein complexes. Replication of telomeric sequences is performed by telomerase, a ribonucleoprotein enzyme complex first identified in the ciliate *Tetrahymena* (13). In humans, this complex is composed of three subunits: (1) the RNA component (hTR), which contains an intrinsic sequence complementary to the G-rich strand of telomeres, thus serving as a template for telomeric repeat synthesis (14); (2) the telomerase-associated protein 1 (TP1), which is similar to the *Tetrahymena* protein component p80 (15,16); and (3) a catalytic subunit, hTERT (17,18).

Telomeres have been suggested to function as a mitotic clock, which indicates to aging cells the time to die. Telomeres from fibroblasts isolated over time from the same individual were found to be shorter at later ages, suggesting that telomeres shorten during the life span of an individual (19). Moreover, when cultured *in vitro*, somatic cells showed a progressive shortening of their telomeres. In fact, about 100 bp of terminal telomeric DNA was lost by normal human somatic cells during each round of replication. Following these experiments, it was hypothesized that immortalization should then be accompanied by an arrest of telomere shortening. Counter and collaborators confirmed this hypothesis when they showed that telomere shortening stopped when cells were immortalized *in vitro* (20).

Analysis of cancerous human cells (using a highly sensitive PCR-based assay called telomeric repeat amplification protocol [TRAP]) (21) revealed that most (90/101) contained telomerase activity, whereas normal somatic cells lacked this activity. These findings have been confirmed, and to date, more than 85% of the 950 primary tumors analyzed thus far exhibit telomerase activity (22). Furthermore, telomerase activity is lost when immortalized cells are induced to differentiate (23).

Because normal human somatic cells have undetectable telomerase activity and most cancer cells have reactivated this enzyme, we hypothesized that reactivation of telomerase during tumorigenicity resulted from the inactivation of a telomerase repressor, which is active in normal somatic cells. If true, we should then be able to suppress telomerase activity in somatic (normal  $\times$  tumorigenic) cell hybrids. To address this hypothesis, we have fused normal human fibroblasts (telomerase negative) with mouse melanoma cells (telomerase positive). These whole-cell hybrids, called BHF, were analyzed for telomerase activity by TRAP. In general, the BHF hybrids had significantly lower telomerase activity when compared to the mouse melanoma parental cell line. Telomerase activity varied from hybrid to hybrid, which was expected because of the heterogeneity and chromosomal instability of whole-cell hybrids. These findings, presented in **Figure 1**, indicated that telomerase activity was under negative regulation in normal somatic cells. By transferring the chromosome(s) that encodes the negative regulator we induced suppression of telomerase activity in the mouse melanoma cell line. To identify the chromosome(s) responsible for the *trans* negative regulation of telomerase activity, we generated a panel of microcell hybrids, (B78MC series), which selectively retain individual tagged human chromosomes (generation and human DNA content of these hybrids is described in **ref. 24**). A subset of these hybrids was analyzed for telomerase activity; some hybrids were found to have completely suppressed telomerase activity (MC series, **Fig. 2**), whereas others had levels similar to that of the B78 parental cell line (e.g., MC63, **Fig. 2**). To identify the chromosomal locus carrying this repressor function, we needed to determine the human chromosome content present in telomerase-negative mouse/human microcell hybrids. To do this, we amplified human-specific sequences (inter-*Alu* sequences) in the microcell hybrids using *Alu* primers 153, 154, 450, and 451 (**25**), followed by biotinylation and fluorescence *in situ* hybridization (FISH) of the PCR products (*Alu*-PCR probe) onto normal human chromosome spreads. The human chromosome(s) hybridizing to the *Alu*-PCR probe therefore represent the one(s) present in the microcell hybrid from which the probe originated. Further confirmation of the

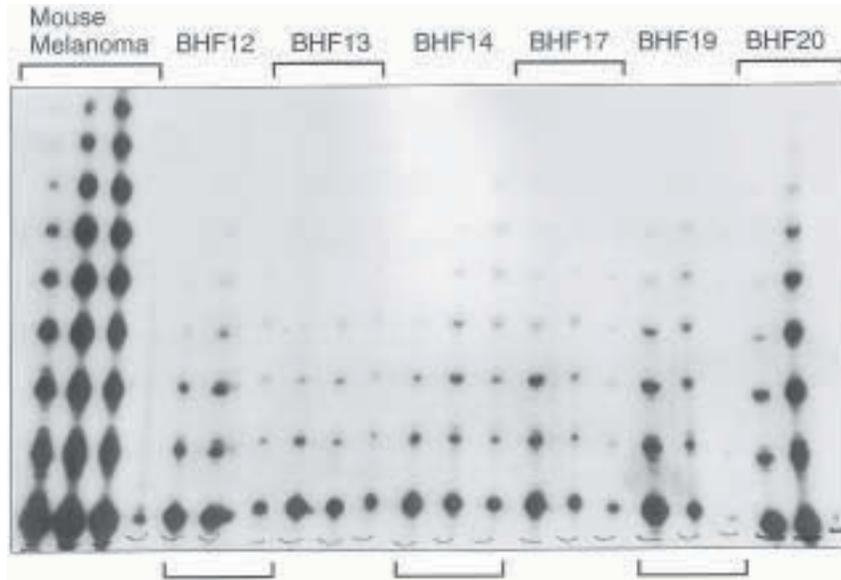


Fig.1. Telomerase activity in BHF whole-cell hybrids. Telomerase activity was measured in cell extracts from individual hybrids by the TRAP procedure essentially as described (21). Amounts of 6, 0.6, and 0.06  $\mu\text{g}$  of protein was assayed for each different hybrid and the mouse melanoma parental cell line. The fourth lane, under mouse melanoma, represents 6  $\mu\text{g}$  of protein treated with RNase prior to the TRAP assay (control to ensure that the bands detected are attributed to telomerase RNA component).

presence of these human chromosomes in the microcell hybrids was obtained by PCR amplification using primers specific for different human chromosomes (available from Research Genetics) and FISH using specific human chromosome painting probes (available from Oncor and Cambio). An example of the human chromosome content in whole-cell and microcell hybrids is shown in **Figures 3** and **4**, respectively.

## 2. Materials

### 2.1. Cell Culture Media

1. Dulbecco's modified eagle medium (DMEM; GIBCO-BRL, 12100-046).

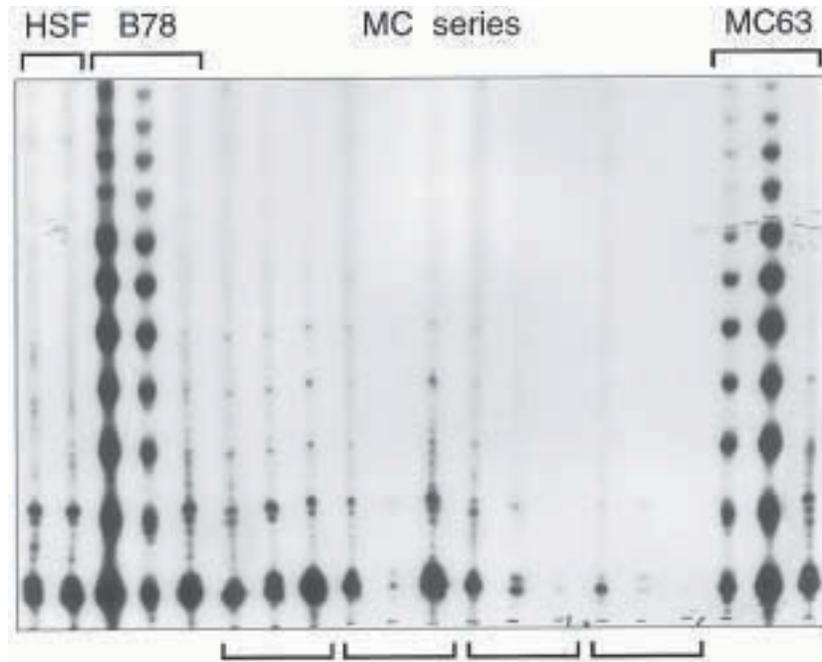


Fig. 2. Telomerase activity in B78 microcell hybrids. The first two lanes are cell extracts prepared from normal human diploid fibroblasts (HSFs, 6 and 0.6  $\mu\text{g}$  of protein, respectively). Lanes 3 and 4 are extracts prepared from mouse melanoma tumor cell line (B78, 6 and 0.6  $\mu\text{g}$  of protein, respectively), and lane 5 is B78 (6  $\mu\text{g}$  of protein) treated with RNase. Microcell hybrids were tested at 6 and 0.6  $\mu\text{g}$  of protein (the third lane of each hybrid represents the RNase-treated control). The results show four different telomerase-negative hybrids and one positive hybrid, indicating that telomerase is under dominant *trans*-negative regulation because of the presence of specific chromosomes and not because of the transfer technique itself.

2. Minimum essential media,  $\alpha$  modification ( $\alpha$ -MEM; GIBCO-BRL, 11900-024).
3. Fetal bovine serum, qualified (FBS; GIBCO-BRL, 10438-018).
4. Penicillin (100 U/mL) streptomycin (100  $\mu\text{g}/\text{mL}$ ) (PenStrep 100 $\times$  solution; GIBCO-BRL, 15140-122).
5. Dulbecco's phosphate-buffered saline (PBS) (10 $\times$  solution; GIBCO-BRL, 14200-075).

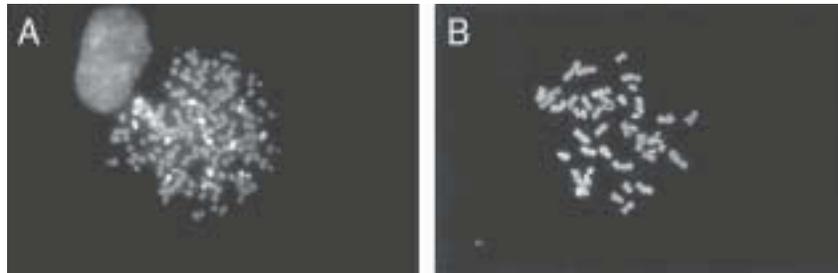


Fig. 3. Human content of whole-cell hybrids. (A) FISH was performed on a BHF12 hybrid metaphase spread using a total human DNA probe. The labeled chromosomes are human (yellow–green fluorescent signal) and the unlabeled chromosomes are mouse (counterstained in red; note that the majority of mouse chromosomes are telocentric and can be distinguished easily from human chromosomes). (B) Biotinylated *Alu* PCR products obtained from BHF12 were hybridized on a normal human metaphase spread. Most of the chromosomes are brightly fluorescent, indicating that this whole-cell hybrid retained most human chromosomes.

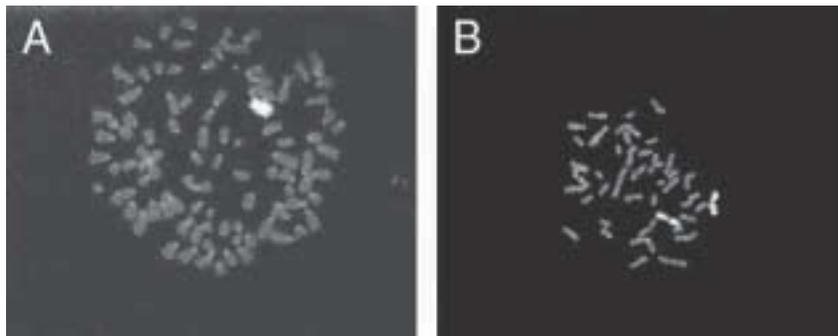


Fig. 4. Human content of microcell hybrids. (A) MC185 microcell hybrid metaphase spread probed with total human DNA probe. The fluorescent signal indicates the presence of only one chromosome. (B) Biotinylated *Alu* PCR products obtained from the same hybrid and hybridized on a normal human metaphase spread. Note that the human chromosome present in the hybrid was not complete with part of the short arm deleted. Because this is a normal spread, two identical chromosomes stained positive.

6. Hygromycin B (50 mg/mL; Roche 843 555).
7. Ouabain (Sigma, 05754).

## **2.2. Cell Fusion**

1. Polyethylene glycol (PEG 1,500) (Code 00-14806/C, New Brunswick Scientific Company, Hatfield, England).
2. Concanavalin A (Con A) (Sigma, C-2631).
3. 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (water-soluble carbodiimide [WSC]; Sigma, C-1011).
4. Colcemid, 1 mg/mL in 0.9% NaCl (Demecolcine) (Sigma, D-7385). This stock solution is stable for a few months when stored in foil-covered tubes at  $-20^{\circ}\text{C}$ .
5. Aceto-orcein: 0.5% orcein (Baker, JTS 756) in 50% acetic acid.
6. Cytochalasin B, 2 mg/mL in dimethylsulfoxide (DMSO) (Sigma, C-6762). The DMSO stock solution is stable for up to a month when stored in foil-covered tubes at  $4^{\circ}\text{C}$ .
7. Phytohemagglutinin P (PHA-P) (Difco Bacto 3110-56).
8. Polycarbonate centrifuge tubes (50 mL) with polypropylene screw caps (Fisher Scientific, 05-563-2C).
9. Nuclepore carbonate filters (5 and 8  $\mu\text{m}$ ) (VWR Scientific, 28158-668 and 28158-806, respectively).
10. Gelman syringe-type holders (25-mm) (Fisher Scientific, 09-730-225). Mount 5- and 8- $\mu\text{m}$  filters in separate filter units. Carefully place the filter between two O-rings, wrap, and autoclave to sterilize.
11. Acrodisc disposable 25-mm syringe filters, Gelman (VWR Scientific, 28144-040).
12. Glass cloning cylinders (Bellco Glass, Inc., 2090-00808).

## **2.3. Human Chromosome Identification**

1. AmpliTaq<sup>®</sup> DNA polymerase with Gene Amp<sup>®</sup> (Perkin Elmer, Roche, N808-167).
2. *C<sub>0</sub>t*-1 human DNA (GIBCO-BRL, 15279-011).
3. PBD (solution containing PBS, bovine serum albumin, and detergent; Oncor).
4. FISH detection kit (Oncor, S1370-CF).
5. Ultrapure dNTP set (Pharmacia Biotech, 272035-01).
6. NuSieve agarose (3/1) (FMC BioProducts, 50090).
7. BioPrime<sup>®</sup> DNA labeling system (GIBCO-BRL, 18094-011).

### 3. Methods

#### 3.1. Whole-Cell Fusion

Somatic cell fusion between cells from different species was first performed more than 30 years ago. This technique was rapidly recognized to be a valuable tool for gene-mapping studies (26) as well as for studying mechanisms of gene regulation (27). The first evidence of tissue-specific negative gene regulation came from studies by Davidson and collaborators (28), who showed that hybrids produced from the fusion of melanoma cells and fibroblasts no longer produced melanin, a specific function of melanoma cells, although the gene encoding this function was present in the hybrids. Because telomerase can be regarded as a tissue-specific function, i.e., being expressed in tumor but not in normal cells, we hypothesized that fusion of a normal cell type with a tumor cell line would lead to hybrids lacking telomerase activity as determined by the TRAP assay. We used normal diploid human fibroblasts (transfected previously with a dual selection marker; described in **Subheading 3.2**) and fused them with mouse melanoma cells. The fusion protocol is described below, and the hybrids obtained were named BHF.

1. Thoroughly mix  $5 \times 10^5$  normal human diploid fibroblasts (HSFs) with  $5 \times 10^4$  mouse melanoma cells (B78) and plate in 25-cm<sup>2</sup> tissue culture flasks (T-25) in DMEM containing 10% FBS and PenStrep (nonselective complete medium). These cell densities were chosen to obtain a mixed monolayer of 80% confluency, and HSFs were used in 10-fold excess, as they have a slow growing rate and do not fuse as well as B78 cells.
2. Remove the medium 24 h after plating, using a Pasteur pipet connected to a suction device (*see Note 1*). Fuse the cell monolayer by adding 1 mL of 50% PEG (*see Note 2*). Tilt the flask back and forth to spread the PEG evenly and incubate under the laminar flow hood for exactly 60 s.
3. Remove the PEG solution and rinse the cell monolayer 3× with serum-free medium. Then add 5 mL of complete (with serum) nonselective medium and incubate cells overnight at 37°C with 5% CO<sub>2</sub> (cell culture incubator).

4. The next day split cells at a 1:20 ratio and plate into selective medium in T-25 flasks (*see Note 3*).
5. Clones should be visible after 14 d. Pick individual clones using glass cloning cylinders (*see Note 4*) and expand in complete selective media. At this point, ouabain can be removed from the selection medium; hygromycin B is always kept in the media to ensure the retention of the introduced tagged human chromosome(s).
6. Measure telomerase activity. Individual clones were tested for telomerase activity using the TRAP assay. Results are shown in **Fig. 1**.

### **3.2. Microcell Fusion**

Microcell fusion is a technique whereby chromosomes from donor cells can be transferred individually into a recipient cell line (31–33). Transferred chromosomes are retained in the recipient cell line under positive selection. This can be achieved by natural means. For example, human chromosome 17 (chromosomal locus for thymidine kinase) can be transferred into a thymidine kinase-deficient cell line and can be retained therein when appropriate selection is used (34). However, natural selection is limited, as only a few human chromosomes contain natural selectable markers. Another and more versatile approach involves the introduction of a bacterial or viral selectable marker. We used this approach to ensure that all human chromosomes were represented in the microcell hybrids for the purpose of gene regulation studies. A dual selection construct containing the hygromycin phosphotransferase (*Hy*) gene fused in-frame with the herpes simplex virus type 1 thymidine kinase (*TK*) gene (30) was used to tag individual human chromosomes in human skin fibroblasts (24). The dual selection cassette confers hygromycin B resistance (through the expression of the *Hy* gene) and ganciclovir sensitivity (attributable to the herpes *TK* gene) to hybrids retaining an introduced tagged human chromosome. Thus, introduced chromosomes can be selectively retained or eliminated from the hybrid cells as desired. The property of dual selection provides formal genetic proof that the observed phenotype is attributable to the introduced human chromosome, as its loss, selected in the presence of ganciclovir, will restore the original phenotype. This approach

was used to identify two novel tumor suppressor loci on human chromosomes 4 and 12 (35,36) and is the strategy being used to identify natural regulator(s) of telomerase activity. The methodology used to obtain a panel of mouse melanoma cells containing individually tagged human chromosomes is detailed below (a broader overview of the technique is described in refs. 33 and 37).

### 3.2.1. Preparation of Bullets

Micronucleation of cells requires high-speed centrifugation. Cells are therefore plated on plastic “bullets” which are treated with Con A to ensure good cell adhesion. Bullets are made from culture dishes cut in size and shape to fit 50-mL polycarbonate centrifuge tubes (see Note 5).

1. Place sterile plastic bullets in a sterile 150-mm plate. Place four to five bullets per plate and air-dry the bullets in a laminar flow hood (bullets should be inverted so that both sides are dried). Make sure that the cell attachment surface is side up and that bullets are not touching each other.
2. Prepare Con A solution at 15 mg/mL in 0.9% NaCl (see Note 6).
3. Prepare WSC crosslinker at 75 mg/mL in 0.9% NaCl (see Note 7).
4. Spread (using a sterile 1-mL culture pipet) 0.6 mL of WSC on the tissue culture surface of each bullet.
5. Using the 1-mL pipet add 0.6 mL of Con A on top of the WSC solution and carefully mix and spread mixture just to edges of each bullet.
6. Incubate 1–2 h in the laminar flow hood.
7. Aspirate the mixture with a Pasteur pipet. Rinse the bullets twice with 20 mL of sterile PBS. Shake the dish to ensure proper rinsing.
8. Bullets are stored at 4°C (placed back to back in 50-mL centrifuge tubes in sterile PBS; up to 6 per tube), or used immediately (see Subheading 3.2.3).

### 3.2.2. Preparation of Donor and Recipient Cells

Recipient cells should be in late log phase, nearing confluency on the day of the fusion. Donor cells are treated with colcemid, a mitotic spindle inhibitor, which induces cells to arrest at M phase. Upon pro-

longed treatment, the cells overcome the cell cycle block and attempt to undergo nuclear division. Lacking chromosomal organization, the nuclei undergo fragmentation, resulting in micronuclei formation. Each micronucleus contains between one and four individual chromosomes (38).

1. Add colcemid to donor cells (HSFs) and incubate for 24–48 h (*see Note 8*).
2. Twenty four hours prior to the microcell fusion, seed sufficient recipient cells (B78) to obtain 90% confluency in T-25 flasks in the appropriate growth medium. Prepare two flasks per fusion (*see Note 9*).
3. The day of the microcell fusion (when good micronucleation has been achieved, i.e., 24–48 h of continuous colcemid treatment), trypsinize micronucleated donor cells, resuspend, pool and centrifuge at 500g. Take an aliquot of cells and count with a hemacytometer.
4. Resuspend cells to a concentration of  $1 \times 10^6$  cells per 1.2 mL of PBS. Stain a sample of cells with aceto-orcein and evaluate the proportion of micronucleated, mononucleated, and mitotic cells (*see Note 10*).

### 3.2.3. Enucleation

In this step, the micronucleated cells are centrifuged in the presence of cytochalasin B, allowing the micronuclei to pop out of the cells, bringing with them a portion of cytoplasm and an intact plasma membrane. These microcells then sediment at the bottom of the centrifuge tube.

1. Place the Con A-treated bullets in a sterile 150-mm Petri dish and add about 1 million micronucleated cells in 1.2 mL of PBS on each bullet (*see Note 11*). Eight bullets (approx 10 million cells) are usually used per fusion. Allow cells to attach to the bullets for approx 15 min. Cover Petri dish and evaluate bullets for cell adhesion by microscopic evaluation.
2. Once cells are attached, add 40 mL of complete medium ( $\alpha$ -MEM containing 10% FBS) and place plates in the incubator until cells have flattened. Check under the microscope every 30 min (cells usually take approx 1<sup>1</sup>/<sub>2</sub> h to adhere).
3. Prepare enucleation medium while waiting for cells to attach (*see Note 12*).

4. Prewarm centrifuge and rotor (SS-34 rotor for Sorvall RC-5B). Centrifuge bullets containing attached micronucleated cells at 27,000g (15,000 rpm) for 30 min at 28–34°C (*see Note 13*).
5. Following centrifugation, check one bullet under the microscope to assess the extent of enucleation (*see Note 14*). Place remaining bullets into a beaker of distilled water to wash them (*see Note 15*). Decant the medium; medium can be poured into a second set of tubes to process another round of bullets (*see Note 16*).
6. Resuspend the cell pellets (obtained from 8 bullets) in 8 mL of serum-free  $\alpha$ -MEM and pour into a 10-mL sterile syringe. Filter sequentially through 8- and 5- $\mu$ m Nuclepore filters. Gently push the suspended preparation through the filters and recover the filtrate (*see Note 17*).
7. Centrifuge the filtered microcell suspension at 2000g for 10 min at room temperature.
8. Resuspend the microcells in 1 mL of serum-free  $\alpha$ -MEM and proceed with the fusion.

### 3.2.4. Fusion

This is very similar to the whole-cell fusion protocol, except that microcells are first incubated in the presence of PHA-P to help them stick to the recipient cell monolayer with which they will be fused.

1. Prepare a 2 $\times$  solution of PHA-P (200  $\mu$ g/mL) (*see Note 18*).
2. Prepare 1 mL/flask of 50% (w/w) PEG in serum-free medium (*see Note 2*).
3. Rinse recipient monolayers with 5 mL/flask of serum-free medium. Add 1 mL of microcell suspension to the fusion flask and 1 mL of serum-free medium to the control flask.
4. Then add 1 mL of PHA-P (2 $\times$ ) to each flask and place in incubator at 37°C for 10–15 min to allow agglutination.
5. Fuse one T-25 flask at a time. Carefully aspirate PHA-P and gently add 1 mL of PEG solution. Incubate under the laminar flow hood for exactly 60 s while rocking the flask gently. At 10 s before the appropriate time, tip the flask back and aspirate the PEG.
6. As quickly as possible, rinse 3 $\times$  with serum-free medium.
7. Add 5 mL of complete (with serum) nonselective medium to each flask and incubate at 37°C with 5% CO<sub>2</sub> overnight (*see Note 19*).

8. The next day split each flask 1/10 or 1/20 into selective medium in T-25 flasks (*see Note 3*).
9. Microcell hybrid clones should be visible after 14–21 d. Pick individual clones using glass cloning cylinders and expand in complete selective medium. At this point, ouabain can be omitted. Hygromycin B must be present to retain the tagged human chromosome.
10. Measure telomerase activity. Individual clones were tested for telomerase activity using the TRAP assay. Results are shown in **Fig. 2**.

### 3.3. Chromosome Identification

The human component of mouse/human microcell hybrids is identified by amplifying human-specific DNA sequences, labeling the PCR products and using these as probes on normal metaphase spreads by FISH. Amplifications are performed using four different *Alu* primers that are set up individually. Following amplification, the PCR products are pooled and labeled with biotin and used as probe for chromosome painting.

#### 3.3.1. *Alu* PCR Amplification

1. Isolate DNA from individual microcell hybrid clones according to standard protocols.
2. Dilute DNA from mouse/human hybrid to 10 ng/μL in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA).
3. Prepare cocktail for PCR procedure: Add 52.5 μL of DNA (10 ng/μL), 52.5 μL of PCR Buffer (10×) (*see Note 20*), 52.5 μL of dNTP (10×) (*see Note 21*), and 3.15 μL of AmpliTaq polymerase (5 U/μL). Add 338 μL of double distilled H<sub>2</sub>O (ddH<sub>2</sub>O).
4. For each DNA sample (mouse/human hybrid clone), set up 10 reaction tubes. Add 47.5 μL of cocktail to each tube followed by 2.5 μL of the appropriate primer (100 ng/μL) (**25**):
  - a. 2 tubes, each containing primer 153 (5'-GGGATTACAGGC-GTGAGCCAC-3')
  - b. 2 tubes, each containing primer 154 (5'-TGCACTCCAGCC-TGGGCAACA-3')
  - c. 2 tubes, each containing primer 450 (5'-AAAGTGCTGGGATTACAGG-3')

- d. 4 tubes, each containing primer 451 (5'-GTGAGCCGAGAT-CGCGCCACTGCACT-3')
5. Mix, microfuge briefly, and place in the thermal cycler.
6. Program thermal cycler as follows (*see Note 22*):

Denature	Anneal	Extend
94°C, 3 min	60°C, 1 min	72°C, 1 min × 1 cycle
92°C, 45 s	60°C, 1 min	72°C, 1 min + 6 s each cycle × 35 cycles
92°C, 45 s	60°C, 1 min	72°C, 10 min

7. Run a 10- $\mu$ L aliquot of *Alu* PCR products on a 2% agarose gel (NuSieve 3:1) to verify successful amplification (*see Note 23*). Pool the remaining 40  $\mu$ L from each tube into a 1.5-mL eppendorf tube.
8. Precipitate pooled *Alu* PCR products (400  $\mu$ L) with 40  $\mu$ L of 3M sodium acetate and 1 mL cold 100% ethanol (EtOH). Incubate at  $-70^{\circ}\text{C}$  for 20 min, thaw, and microcentrifuge for 15 min. Remove supernatant and wash with cold 70% EtOH. Microcentrifuge and remove supernatant. Dry pellet and resuspend in 50  $\mu$ L of TE buffer. Quantitate.

### 3.3.2. Labeling of *Alu* PCR Products

The human *Alu* PCR products representing all of the human sequences present in the microcell hybrids are labeled with biotin-dUTP using a commercially available labeling kit.

1. Denature 100 ng of *Alu* PCR DNA products by heating for 5 min in a boiling water bath. Cool on ice immediately. (The amount of template per reaction can be varied from 25 to 500 ng with satisfactory results.)
2. Perform the following additions on ice: 5  $\mu$ L of 10 $\times$  dNTP mixture, 20  $\mu$ L of 2.5 $\times$  random primer solution (BioPrime DNA labeling kit), and complete with ddH<sub>2</sub>O to a total volume of 49  $\mu$ L. Mix.
3. Add 1  $\mu$ L of Klenow fragment. Mix gently but thoroughly. Centrifuge 15–30 s.
4. Incubate at 37°C for 60 min.
5. Add 5  $\mu$ L of stop buffer.
6. Perform EtOH precipitation by adding one-tenth vol of 3M sodium acetate and 2 vol cold 95% (or absolute) EtOH. Mix by inversion

and precipitate at  $-70^{\circ}\text{C}$  for 15 min or at  $-20^{\circ}\text{C}$  for 2 h. Thaw and centrifuge at  $15,000g$  for 10 min. Remove the supernatant carefully and dry the pellet. Resuspend in  $10\ \mu\text{L}$  of water. (The concentration should be about  $100\ \text{ng}/\mu\text{L}$ ). Store at  $-20^{\circ}\text{C}$ .

### 3.3.3. FISH

The biotin-labeled *Alu* PCR fragments are used as a probe on normal human chromosome spreads.

1. Prepare normal human chromosome slides as per standard cytogenetic procedures. Make slides and age overnight on a  $55^{\circ}\text{C}$  slide warmer. Slides can be stored at  $-20^{\circ}\text{C}$  for several weeks.
2. Prepare denaturation solution (70% formamide in  $2\times$  SSC): 28 mL of formamide, 4 mL of  $20\times$  SSC (see **Note 24**), and 8 mL of  $\text{ddH}_2\text{O}$ .
3. Fill a Coplin jar with denaturation solution and place in a water bath at  $75^{\circ}\text{C}$ .
4. Meanwhile, dehydrate slides sequentially, 2 min each time, in ice-cold EtOH—70%, 90%, 100%. Air-dry slides. Using phase-contrast microscopy, examine slides and select slide area for hybridization (there should be adequate quantity and quality of chromosome spreads).
5. Prepare competition hybridization mixture. Per slide, set up in a 0.5-mL Eppendorf tube: 3  $\mu\text{L}$  of human *C<sub>0</sub>t-1* DNA ( $1\ \mu\text{g}/\mu\text{L}$ ), 1  $\mu\text{L}$  of 100 ng of biotinylated *Alu* PCR products, 1  $\mu\text{L}$  of sheared salmon sperm DNA ( $1\ \mu\text{g}/\mu\text{L}$ ), 1  $\mu\text{L}$  of  $\text{ddH}_2\text{O}$ , 10.5  $\mu\text{L}$  of master mix (see **Note 25**) to a total volume of 16.5  $\mu\text{L}$ .
6. Microcentrifuge competition hybridization mix briefly. Denature in  $75^{\circ}\text{C}$  bath for 10 min. Transfer immediately to a  $37^{\circ}\text{C}$  bath to pre-anneal for 30 min.
7. Denature slides in denaturation solution (**step 2**) for 2 min at  $70$ – $72^{\circ}\text{C}$  (see **Note 26**).
8. Transfer slides to 70% EtOH (on ice) immediately and dehydrate as in **step 4**. Air-dry.
9. Once the hybridization mixture has preannealed, microcentrifuge briefly and apply full amount (16.5  $\mu\text{L}$ ) to the chosen location on the slide.
10. Apply a  $22 \times 22$  mm glass coverslip and seal with rubber cement.
11. Incubate overnight at  $37^{\circ}\text{C}$  in a humidified chamber (see **Note 27**).

### 3.3.4. Detection

The *Alu* PCR probe hybridizes to specific human chromosomes represented in the source microcell hybrid. Hybridization is detected using an avidin/anti-avidin antibody detection system (Oncor or Cambio). The green fluorescent hybridization signal is observed using a fluorescent microscope with the appropriate filters for visualization of the hybridization signal and the counterstain.

1. Prepare posthybridization wash solutions (*see Note 28*). Make 40 mL of each posthybridization wash solution and pour into Coplin jars. Heat to 43–45°C before using.
2. Remove slides from moisture chamber and remove the coverslip sealant from each slide. Dip the slide in posthybridization wash solution 1 and shake gently to loosen coverslip. Remove coverslip from the slide and leave the slide in solution.
3. Wash the slides in a 43–45°C shaking water bath:
  - a. Wash solution 1, 10 min.
  - b. Wash solution 2, 10 min.
  - c. Wash solution 3, 5 min.
4. Transfer to 1× PBD and proceed with detection using the Oncor detection kit. Remove slides from PBD and immediately add 30 µL of FITC–avidin to the chosen location on the slide. Cover with a plastic coverslip and incubate 20 min at 37°C.
5. Wash in PBD three times for 2 min each at room temperature.
6. Amplify signal by adding 30 µL of avidin antibody. Cover with plastic coverslip and incubate at 37°C for 5–20 min.
7. Wash in PBD three times for 2 min each at room temperature.
8. Add 30 µL of FITC–avidin to bind to avidin antibody, cover with plastic coverslip, and incubate at 37°C for 5–20 min.
9. Wash in PBD three times for 2 min each at room temperature.
10. Stain with 15 µL of propidium iodide diluted in antifade solution according to manufacturer's conditions.
11. Observe under fluorescence microscope and photograph good chromosome spreads.

## 4. Notes

1. Before adding the PEG solution, it is very important to remove all of the medium so as not to dilute the solution. This is done by first

removing the medium by aspiration, waiting for 20–30 s with the flask in a vertical position to allow remaining medium to drain down, and then aspirating residual medium.

2. The type and grade of PEG used is very important. PEG 1,500 was used here and was obtained from New Brunswick Scientific Company (*see Subheading 2.2, step 1*). PEG is prepared in serum-free media and warmed at 37°C. The solution is then sterilized by filtration through a 0.22- $\mu$ m filter. It is important to note that the concentration of the PEG solution is expressed on a weight/weight (w/w) basis, assuming that 1 mL of media weighs 1 g. Thus, a 50% solution would be prepared by using 5 g of PEG plus 5 mL of serum-free media. A small variation in PEG concentration and incubation time can affect hybridization efficiency (29). Different cell lines might require different PEG concentrations and incubation periods. These conditions must be determined empirically for each cell line.
3. The human diploid fibroblasts were tagged with a dual selective marker, tgCMV/HyTK (24,30). These cells grow in the presence of 400  $\mu$ g/mL hygromycin B, which is toxic to the untransfected B78 cells. Human cells are killed in 3  $\mu$ M ouabain, a concentration that does not affect rodent cells. Therefore, only hybrid cells will survive in the presence of hygromycin B and ouabain. The selection media used was DMEM, 10% FCS, 400  $\mu$ g/mL hygromycin B, 3  $\mu$ M ouabain, and PenStrep.
4. As cell clones become visible (usually 50 cells), they are harvested and expanded. The top portion of the culture flask is cut out using a soldering gun. Autoclaved cloning cylinders are first dipped in autoclaved grease (centrifuge vacuum grease was used which was autoclaved in a glass Petri dish) and then deposited around the cells. A few drops of trypsin are added, and after 1 or 2 min the cells are harvested using a sterile cotton-plugged Pasteur pipet.
5. Bullet-shaped pieces of plastic are cut from tissue culture plates using a hot-wire cutter. The bullets are approx 24  $\times$  86 mm and have one rounded end. They fit, round end down, into 50-mL polycarbonate centrifuge tubes. The bullets are stored and sterilized by soaking in 95% ethanol (2 h minimum, preferably overnight).
6. Incubate the Con A solution in a 37°C water bath for about 30 min; it will not be entirely soluble. Filter by gravity through Whatman no.1 paper and then sterilize through a 0.22- $\mu$ m filter.
7. This is readily soluble. Prepare and filter sterilize through a 0.22- $\mu$ m filter just before use.

8. Cells should be in exponential growth when the colcemid is added. The effective colcemid concentration for micronucleation varies with the cell line. Phase-contrast examination of the treated cultures is usually sufficient to define the concentration of colcemid required. Rodent cells require between 0.01 and 0.1  $\mu\text{g}/\text{mL}$ , whereas human cells need much higher concentrations (10–20  $\mu\text{g}/\text{mL}$ ). Use 0.06  $\mu\text{g}/\text{mL}$  for mouse melanoma cells (B78) and 10  $\mu\text{g}/\text{mL}$  for HSFs.
9. Prepare a flask of recipient cells for control fusion. Do not add microcells to this flask (only PHA-P in serum-free medium and fuse with PEG). No clones should appear in this fusion. This ensures that the selection medium is appropriate.
10. Mix a drop of microcell preparation with a drop of aceto-orcein solution on a microscope slide and overlay with a coverslip. Observe under the microscope using transmitted light.
11. Add the cells to the bullets using a 2-mL sterile pipet. Use the pipet to spread the cell suspension evenly on the surfaces of the bullets.
12. Enucleation buffer: prepare 500 mL of serum-free  $\alpha$ -MEM containing 10  $\mu\text{g}/\text{mL}$  cytochalasin B (2.5 mL of 2 mg/mL stock; *see Subheading 2.2, step 6*) for 8 tubes. Pour about 40 mL into each of 8 sterile polycarbonate 50-mL centrifuge tubes with lids.
13. It is possible to spin two bullets per tube, placed back to back with cell sides facing outward. The bullets are oriented sideways in the centrifuge tube. When one bullet is used, it is oriented with the cell side facing the outside of the rotor.
14. Observe a bullet under the phase microscope immediately after removing it from the tube. Streaks of cytoplasm and debris indicate good enucleation. Incompletely enucleated monolayers have micronuclei extending from the cell bodies like beads on a string.
15. After enucleation, remove the bullets from the tubes and place in distilled water. Rinse bullets thoroughly and store in 95% ethanol for further use.
16. Alternatively, the cytochalasin B serum-free media solution can be filter sterilized and kept at 4°C. This solution can be reused once or twice and is stable for up to 6 mo.
17. Gently push the syringe plunger to filter the solution so as not to clog or break the filter. Allow the solution to filter through by gravity as much as possible. Be careful to work aseptically because these filters do not remove bacteria. This step is performed to reduce contamination of whole cells and enrich microcells.

18. Prepare a solution using 2 mg of PHA-P (the commercially available powder is at 50% concentration) and 5 mL of serum-free  $\alpha$ -MEM (1 mL of 2 $\times$  PHA-P solution is required per T25 flask of recipient cells). Filter sterilize through a 0.22- $\mu$ m filter.
19. This overnight incubation, in the absence of selection, allows the cells to recuperate from the fusion and permits the expression of the selectable marker encoded by the bacterial gene.
20. PCR buffer (10 $\times$ ): 500 mM KCl, 100 mM tris-HCl (pH 8.4 at 20°C), 15 mM MgCl<sub>2</sub>, and 1 mg/mL gelatin. This buffer is provided with *Taq* polymerase.
21. dNTP (10 $\times$ ): 2 mM dATP, 2 mM dCTP, 2mM dGTP, 2 mM dTTP (10 mM individual dNTPs stock from Pharmacia).
22. Program the thermal cycler to add 6 s to the extension time in each cycle, so that the first extension time is 1 min, the second is 1 min and 6 s, the third is 1 min and 12 s, etc.
23. The agarose gel should reveal a smear of DNA with some repetitive elements characteristic of each primer used. The best smears are usually obtained with primer 451; however, all PCR products are pooled.
24. 20 $\times$  SSC is 3M NaCl plus 0.3M sodium citrate, pH 7.0.
25. Master mix: Make stock with 5 mL of formamide, 1 g of dextran sulfate, and 1 mL of 20 $\times$  SSC. Dissolve by heating to 60°C and mixing well.
26. The water bath should be at about 75°C so that the denaturation solution in the Coplin jar is about 72°C. The temperature should not drop below 70°C, so it is best to warm the slide prior to putting it into the denaturation solution. It is also recommended not to allow the temperature to climb too high, as this could affect chromosome morphology.
27. Use a plastic box or Petri dish in which a Whatman paper previously soaked in water has been placed. Support the slides on two wooden toothpicks to ensure that they do not touch the wet paper.
28. Wash solutions are described as follows:
  - a. Posthybridization wash 1: 50% formamide/2 $\times$  SSC (20 mL of formamide, 4 mL of 20 $\times$  SSC, 16 mL of H<sub>2</sub>O).
  - b. Posthybridization wash 2: 2 $\times$  SSC.
  - c. Posthybridization wash 3: 4 $\times$  SSC/5% Triton X-100 (8 mL of 20 $\times$  SSC, 2 mL of Triton X-100, 30 mL of H<sub>2</sub>O).

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## Screening with COMPARE Analysis for Telomerase Inhibitors

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### 1. Introduction

Modern biology provides a plethora of experimental evidence that points to the chaotic and heterogenous nature of cancer, making it necessary to tackle each type of cancer as a unique problem in terms of treatment. The existence of a labyrinth of multiple backup networks for every biological event in the living cell, from membrane receptors and signal transduction proteins to cell cycle systems and DNA/RNA processing machinery, performs as a vicious snare when it comes to the treatment of cancer. Alternatively, the huge number of chemotherapeutic options available today, with all of their known and unknown pharmacological modes, presents another sort of chaos and heterogeneity. Taken together, the achievement of improved cancer therapy would be easier if we applied our modern knowledge of informatic sciences as a means of managing and matching the two sources of heterogeneity or chaos, i.e., cancer and chemotherapy.

Disease-orientated screening (DOS) is a strategy based upon the correlation of in vitro growth inhibition activities of tested compounds against a panel of various human cancer cell lines, corresponding to

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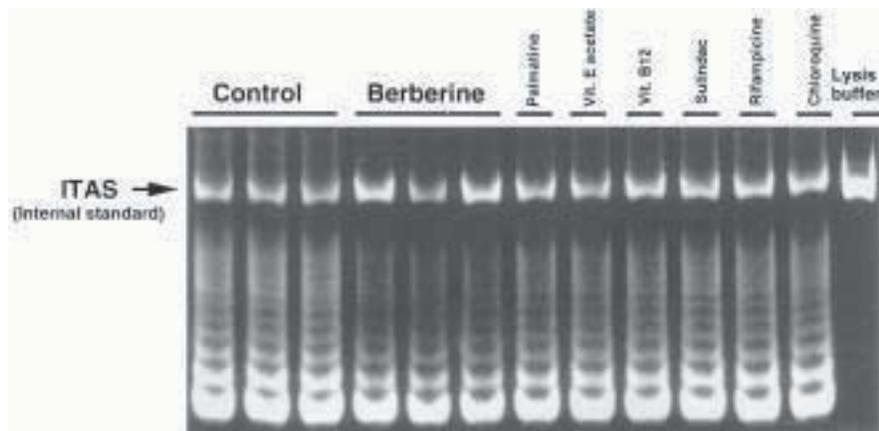


Fig. 1. Screening of telomerase inhibition by randomly selected compounds at 30  $\mu$ m.

clinical cancer types. The concept of this screening was recently adopted (1) in a manner similar to that ongoing at the National Cancer Institute (NCI) (2–4), in the hope of combating the heterogenous nature of cancer. Our screening database consists of the growth inhibitory effects of more than 1000 agents against a panel of 38 human cancer cell lines (lung, ovary, CNS, stomach, colon, kidney, melanoma, and breast) and is therefore likely to cover all possible pharmacological trends in cancer. The growth response data (the concentration of an agent causing a 50% inhibition of growth when compared to the control or  $GI_{50}$ ) as transformed from numerical values to graphic patterns (mean graphs or fingerprints) originally according to the method of Paull et al. (2).

What has emerged from the accumulation of the growth inhibitory data, distinct from the initial aim of the screening program, is that the application of informatic algorithms on the resulting database can clearly discern compounds with novel mechanisms of action. This chapter here demonstrates how one can apply COMPARE analysis (5) successfully as an informatic approach to identify agents with strong inhibitory effects against the potential antitumor target telomerase (6–9).

For example, in a preliminary random screen we identified the alkaloid berberine as a moderate inhibitor ( $GI_{50}$  of approx 35  $\mu\text{m}$ , **Fig. 1**). Using this alkaloid as a seed compound in COMPARE analysis, other berberine-like compounds have been selected, with the mitochondrion-accumulating agents being the most similar to berberine. Among these compounds, MKT077, a rhodacyanine derivative currently in phase-1 clinical trials (**10**), demonstrated a potent inhibitory effect, with a  $GI_{50}$  of approx 5  $\mu\text{m}$ . Using MKT077 as an upgraded seed in a new round of COMPARE analysis, we can select FJ5002, a derivative of MKT007, as a potent inhibitor of telomerase ( $GI_{50}$  of approx 2  $\mu\text{m}$ ). Further work has confirmed that FJ5002 is a genuine and effective antitelomerase agent (**11**).

## 2. Materials

1. Cell line panel composed of 38 cell lines from the 8 different tissues (**Table 1**), characterization described elsewhere (**11,12**).
2. All rhodacyanines tested were supplied by Fuji Photo Film Co. (Kanagawa, Japan), dissolved in dimethylsulfoxide and stored at  $-80^{\circ}\text{C}$  prior to use.
3. All other agents (vincristine, taxol, berberine) were already in clinical use and obtained from Sigma or Takara (Japan).
4. 96-well culture plates.
5. Trichloroacetic acid (TCA), 50% in  $\text{dH}_2\text{O}$ .
6. Sulforhodamine B, 0.4% dissolved in 1% acetic acid.
7. All water used pretreated with diethylpyrocarbonate (DEPC).
8. Tris-HCl, 10 mM (pH 10.5).
9. Cell lysis buffer (all purchased from local suppliers at highest purity available): 10 mM Tris-HCl (pH 7.5), 1 mM  $\text{MgCl}_2$ , 1 mM EGTA, 0.5% (w/v) CHAPS, 10% (v/v) Glycerol, and 5 mM  $\beta$ -mercaptoethanol (AEBSF).
10. Culture medium: RPMI 1640 (Nissui Co. Ltd, Japan), 100  $\mu\text{g}/\text{mL}$  kanamycin, 5% fetal bovine serum.
11. 10 $\times$  PCR buffer: 200mM Tris-HCl, pH 8.3, 15 mM  $\text{MgCl}_2$ , 680 mM KCl, 0.5% (v/v) Tween 20, and 10mM EGTA.
12. TS and CXext primer custom synthesized by Sawady Technology, Tokyo, Japan. Dissolve in DEPC water and store at  $-20^{\circ}\text{C}$  prior to use.

**Table 1**  
**Cell Panel used in the DOS Program**

Organ	Cell Line	Organ	Cell Line
Colon	HCC2998	CNS	U251
	KM-12		SF-268
	HT-29		SF-295
	WiDr		SF-539
	HCT-15		SNB-75
	HCT-116		SNB-78
Breast	HBC-4	Kidney	RXF-631L
	BSY-1		ACHN
	HBC-5	Ovary	OVCAR-3
	MCF-7		OVCAR-4
	MDA-MB-231		OVCAR-5
Lung	NCI-H23	Stomach	OVCAR-6
	NCI-H226		SK-OV-3
	NCI-H522		St-4
	NCI-H460		MKN1
	A549		MKN7
	DMS273		MKN28
	DMS114		MKN45
Melanoma	LOX-IMVI		MKN74

13. TRAP reaction buffer (43  $\mu$ L/reaction): 28.3  $\mu$ L of DEPC water, 5.0  $\mu$ L of 10 $\times$  PCR buffer, 1.0  $\mu$ L of 2.5 mM dNTP mixture, 2.0  $\mu$ L of TS primer (8.6  $\mu$ M), 0.2  $\mu$ L of T4g32 protein (5  $\mu$ g/mL) (Boehringer), 0.5  $\mu$ L of Gene *Taq* DNA polymerase (5  $\mu$ /mL), and 6.0  $\mu$ L of internal telomerase assay standard (ITAS) (0.05 pM).
14. U937 human leukemia cell extract, equivalent of 1000 cells/2  $\mu$ L.
15. Eppendorf microcentrifuge.
16. Perkin-Elmer 9600 thermal cycler.
17. CXext primer, 8.6 mM.
18. 8  $\times$  10 polyacrylamide gel electrophoresis (PAGE) system.
19. Nondenaturing polyacrylamide gel (10–12.5%).
20. Tris-boric acid, EDTA buffer (TBE) at pH 8.0 (Sigma).
21. SYBR Green nucleic acid stain, used at 1:10,000 in 1 $\times$  TBE buffer (Takara, Tokyo, Japan).
22. Gel analysis equipment such as the Bio-Rad MultImager.

### 3. Methods

#### 3.1. Chemosensitivity and COMPARE Analysis (see Notes 1 and 2)

1. Perform chemosensitivity test in 96-well plates using a 48-h automated assay with sulforhodamine B (**Fig. 2**), as described previously (**13**).
2. Forty-eight hours after treatment with the agent of interest, add 50  $\mu\text{L}$  of cold 50% TCA to produce a final concentration of 10%.
3. Wash five times with tap water and dry the plates carefully.
4. Add 50  $\mu\text{L}$  of 0.4% sulforhodamine B.
5. Incubate for 10 min at room temperature, rinse five times with 1% glacial acetic acid, and then dry the plates.
6. Add 150  $\mu\text{L}$  of Tris-HCl.
7. Agitate the plates for 5 min and then measure the absorbance at 525 nm.
8. Determine the growth response at four logarithmic points of drug concentration. Data are represented as the  $\text{GI}_{50}$ , the main measure of chemosensitivity, and graphically transformed to mean graphs or fingerprints using the method of Paull et al. (**2**) (**Fig. 3**).

#### 3.2. Detection of Telomerase Activity in Cell Lysates (see Notes 3–5)

1. Perform the TRAP assay as described previously (**14,15** and elsewhere in this volume), with the addition of ITAS (**16**) and a modified CX primer CXext (**17**). Carry out all procedures on ice.
2. Use the U937 cell line as a standard source of telomerase (derived from one stock culture). Passage at a density of  $5 \times 10^4$  cells/mL in 10-cm tissue culture dishes every 3 days.
3. Add 5  $\mu\text{L}$  of 10 $\times$  drug solution; then add 2  $\mu\text{L}$  of U937 cell lysate (adjusted to the equivalent of  $10^3$  cells). Mix by careful pipeting several times.
4. Spin the tubes briefly at maximum speed in an Eppendorf microcentrifuge and then incubate in a thermal cycler at 20°C for 30 min.
5. Remove the tubes and keep on ice. Add 2  $\mu\text{L}$  of CXext primer and mix by careful pipeting.
6. Spin the tubes briefly at maximum speed in a Eppendorf microcentrifuge and start thermal cycling using the following settings: 94°C

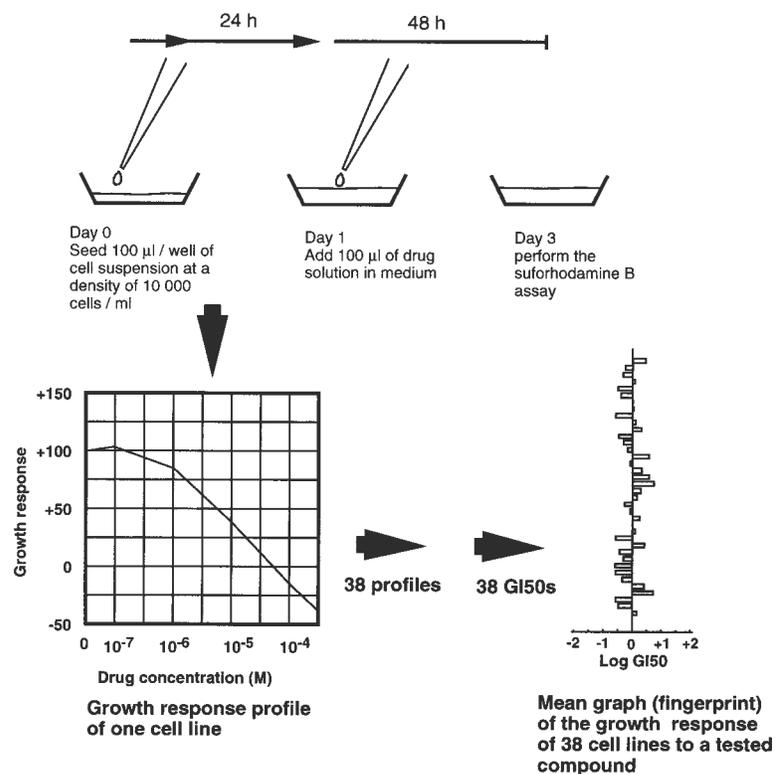


Fig. 2.

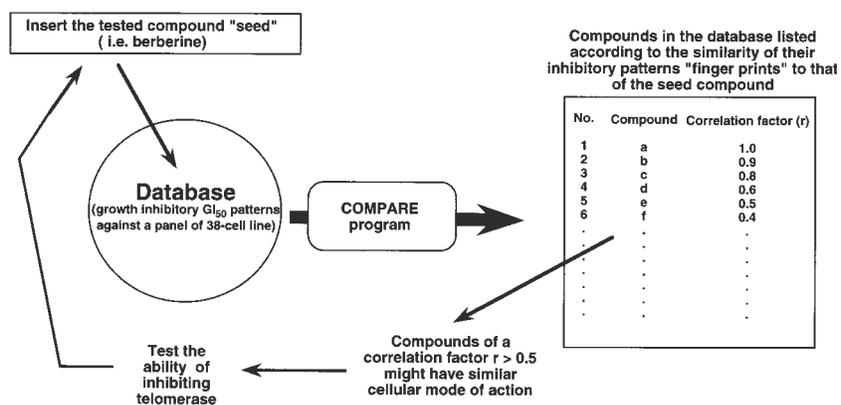


Fig. 3. Simplified overview of the screening approach to the identification of telomerase inhibitors by COMPARE analysis.

for 2 min and 30 s, 94°C for 25 s, 56°C for 25 s, 72°C for 45 s, (repeat the 94°C/56°C/72°C cycle 30×), and 72°C for 2 min.

7. Hold the tubes at 4°C until analysis by PAGE.

### **3.3. Analysis of Telomerase Activity (see Notes 6–10)**

1. Resolve telomerase products by applying 15 µL/lane of PCR product on a nondenaturing polyacrylamide gel.
2. Visualize products by staining with SYBR Green and examine with 250 or 300 nm UV illumination.
3. To achieve quantitative measurements, optimize the reaction conditions in the preliminary experiments to be in a dynamic range with a >0.99 correlation between the signal (log) and the number of cells in the extract (log).
4. Carry out calculations via densitometric analysis using software such as NIH Image 1.60.
5. Divide the densitometric value of the telomerase activity signal by the densitometric value of the ITAS to normalize the values for possible PCR amplification differences.

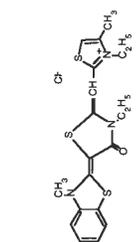
## **4. Notes**

1. The use of different assay methods or growth inhibition criteria could yield substantially different inhibition profiles and, hence, different correlation data among the tested agents.
2. The growth response after a 48-h treatment does not reflect toxicity built up at the lineage level. This property is expected in the case of specific telomerase inhibitors in which their toxicity would only appear after several cellular divisions and resulting telomere shortening. The strategy of screening presented here is believed to be effective with telomerase inhibitors because it identifies molecules with inherent similarity of interaction with the same molecular spectrum in the living cell.
3. Cell extraction with the CHAPS lysis buffer should be accompanied by vortex mixing (two to three times), 10 s each, to ensure complete extraction.
4. To avoid pH changes in the reaction/compound mix, tested compounds should be prediluted in the same buffer used in the TRAP reaction.

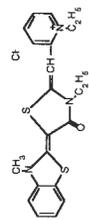
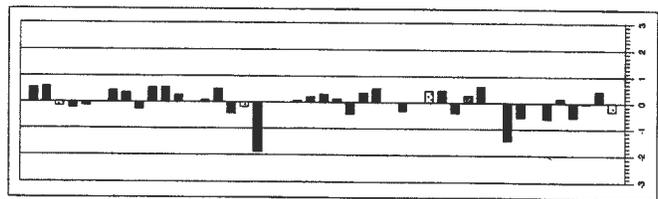
5. When calculating the ladder density of telomerase activity, after capturing the gel image to NIH Image 1.60, a background line should be drawn beyond all the lanes examined. This ensures that the density attributable to the line at one lane is equal to that in other lanes, therefore avoiding differences caused by the arbitrary units of density.
6. In the case of a telomase inhibitor, the correct seed for COMPARE analysis should be any compound included in the database that has even minimal inhibitory effects against telomerase. After testing of approx 30 diverse molecules, berberine chloride could be detected as a moderate inhibitor (**Fig. 1**).
7. When berberine is used as an initial seed in COMPARE analysis, other berberine derivatives and the mitochondrial accumulating agents are distinguishable, having similar growth response “fingerprints” (**Fig. 4**). The testing of the telomerase inhibitory effect of the top 20 correlated agents ( $r > 0.6$ ) has selected MKT077 as a potent inhibitor.
8. Another round of analysis using the upgraded seed MKT077 identifies the rhodacyanine FJ5002 as a more potent telomerase inhibitor (**Fig. 4**). Using this as a new seed in a third round of analysis yields a repeated list of correlated compounds, all of which have already appeared in the first and second rounds. This indicates that FJ5002 is the strongest inhibitor available in the database.
9. When various modifications of berberine were examined, none were found to exhibit significant telomerase inhibition. This indicates that the strategy of using DOS and COMPARE is very helpful in facilitating the screening for effective inhibitors.
10. The continued passage of human cancer cells in the presence of sublethal concentrations of FJ5002 has produced concentration-dependent telomere erosion and increased chromosome abnormalities and crisis/senescence-like features (**II**).

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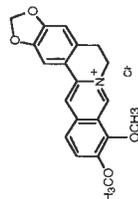
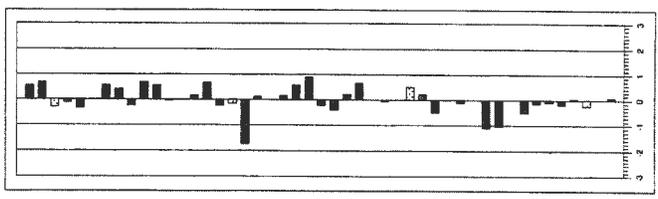
Fig. 4. (*opposite page*) (A) Comparison of the inhibitory effects of berberine, MKT077, and FJ5002 on the activity of telomerase at 10  $\mu$ M. Each value is the mean + SD. (B) Mean graphs of cell growth inhibition by the selected telomerase inhibitors.



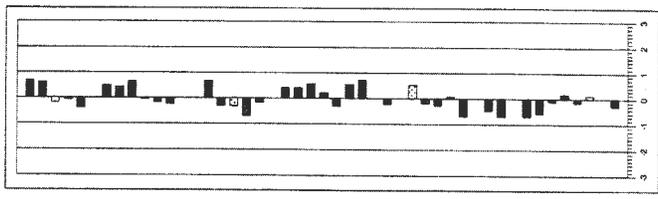
**FJ5002**



**MKT077**

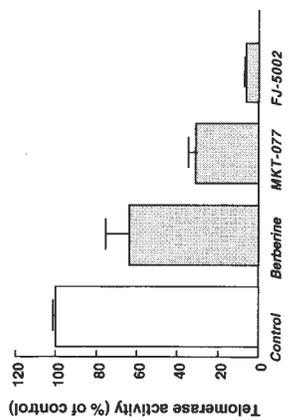


**Berberine**



**B**

**A**



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## Telomerase as a Therapeutic Target

### *Therapeutic Potential of Telomerase Inhibitors*

**John A. Double**

The most desirable properties of any therapeutic agent must be selectivity and potency (*I*). Of the two, potency is less critical, though in terms of potential administration it is important to recognize that patients should not be expected to take grams of material nor can the costs of producing large quantities be ignored.

In general terms, selectivity may arise from two main areas: the uniqueness of the target or the technology of drug delivery. It is the ratio of damage to the specific target to damage in normal tissues that gives rise to the therapeutic index; ideally, this should be as large as possible. In infectious diseases, for example, the target, perhaps the bacterial cell wall, is highly specific to the invading organism, i.e., one not found in the host, therefore, providing the opportunity for selectivity. In general, antibiotics have a very high therapeutic index, and side effects are not the result of target interactions in normal tissues. With standard anticancer agents, toxic side effects, which all reduce the therapeutic index, are the results of target interaction in normal tissues. The technology of drug delivery may still have a significant role in improving the therapeutic index of standard agents; however, it is now generally accepted that we need to move away

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from targets that are principally associated with cell proliferation and try to find some that may be more specific to the cancer process.

The initial findings by Kim et al. (2), showing that telomerase was present in many cancer cell lines and not apparently active in “normal” cells, raised the hope that at last a target, specifically associated with the cancer process, had been found. Since then a substantial body of information has been accumulated, much of which suggests that cancer therapies based on telomerase inhibition could be more effective and safer than current approaches (3–11).

In assessing the potential of telomerase as a therapeutic target and the potential of telomerase inhibitors as anticancer agents the following comments will focus around what is considered the exploitability of the target itself and the desirable properties of therapeutic agents. There is no doubt that telomerase is expressed in the majority of cancers. If this expression was specific to cancers, it would be an ideal target for exploitation and one would be very optimistic that with the current expertise in drug design and synthesis, in the near future new agents with desirable therapeutic properties would emerge. However, it has also become clear that telomerase expression is not unique to the cancer process (4) and that careful design of therapeutic regimens based on telomerase inhibition will be required if toxicity to stem cells, for example, is to be kept to a minimum. Nevertheless, Holt et al. (5) had argued previously that even if this were the case, there would still be a window of opportunity for therapeutic intervention and there was a good case to develop antitelomerase agents. These investigators present the case that because of the lack of telomerase activity in normal cells, their telomeres will have become significantly shorter than those in germ cells and stem cells, which would have been maintained by telomerase activity by the time telomerase was activated in the cancer process. Thus, although an antitelomerase agent may not repress telomerase selectively in cancer cells, the effect of such repression would be far greater in cancer cells and would lead to their growth arrest before this occurred in normal cells. Furthermore, it is possible that, a regulatory mechanism exists to specifically maintain germ-line and stem cell telomere length, which could restore their telomere lengths after

the cessation of telomerase inhibition therapy. This model would clearly support the case for developing agents to inhibit telomerase activity. Although this may be a good model on which new therapeutic strategies could be based, it does rather oversimplify the situation. For example, it has been calculated that inhibition *per se* will not lead to cell death in a time span that is relevant to the treatment of cancer (3), although Harley and colleagues (12) point out that these calculations do not take into account that only a small fraction of cells may be proliferating in many tumors.

The report by Burger et al. (13) highlights several problems associated with the development of any new therapeutic agent that would need to be overcome before a telomerase inhibitor could be brought into clinical practice. The data confirmed that high to moderate levels of telomerase were present in both human and murine tumor tissue, perhaps supporting the view that telomerase could be a suitable target for therapeutic intervention. However, the levels of activity found in murine liver were often higher than those in murine tumors. This may be a potential problem in using murine models in drug development programs as even with a specific inhibitor, it might be very difficult to demonstrate selectivity and a good therapeutic index. Alternative animal systems are invariably more expensive, and in this limited study they also showed that high levels of telomerase activity were also present in vital organs of other species. This study concluded that with the proposed function of telomerase, even complete enzyme inhibition would not produce acute cell death; only cell senescence and subsequent cell death would result from progressive telomere shortening during successive cell divisions. This greatly complicates the assessment of inhibitors as potential anticancer agents, as tumors will continue to grow in size following complete and selective inhibition of telomerase activity. Thus a "perfect" inhibitor may appear inactive in many of the experimental animal model systems currently used; further more, unless it becomes possible to target only telomerase in tumors, therapeutic indices will be very small and the inhibitor may not show any antitumor activity other than at maximum tolerated doses. However, it is believed that with novel strate-

gies and combinations with other modalities of cancer treatment, the development of telomerase inhibitors are still worthwhile.

The development of an active telomerase inhibitor must be well within the bounds of modern drug design, and there are several approaches under investigation. Strahl and Blackburn (*14,15*) have shown that certain inhibitors of reverse transcriptase can reduce telomerase activity and induce telomere shortening. It has also been demonstrated that targeting the telomerase RNA component (hTR), by use of approaches such as antisense oligonucleotides or peptide nucleic acids, can also inhibit telomerase activity in cell-free systems and may therefore represent a viable approach for rational drug design (*8,9,16–19*), though delivery of such agents remains problematic (*4*) and results are sometimes not promising (*20*).

It has been suggested that targeting the guanosine-rich regions of the telomeric tandem repeat sequences, the hTR component, and its gene might be another potential approach for drug design (*16*). To a certain extent the potential of a sequence-selective approach was demonstrated in the report by Burger et al. (*21*). In this report, cisplatin, an agent known to target guanosine-rich regions of DNA (*22*) but not other DNA damaging agents, inhibited telomerase activity in testicular tumor cell lines. Other studies have suggested that this effect may be either cell specific (*23–25*) or may be dependent upon telomere length (*26*). It remains conceivable that among the myriad of platinum agents that have been tested for their antitumor activity there may be a very good telomerase inhibitor; however, with the experimental chemotherapy protocols used in this process, it is unlikely that it would have produced sufficient antitumor activity to warrant further investigation and development. The potential of some of these platinum agents as telomerase inhibitors is currently under investigation (D.R. Newell, personal communication). Further knowledge of the cell biological mechanisms underlying telomerase activity (*5,27–31*) and telomeres (*32–34*) may provide insight into other therapeutic targets.

Telomerase and telomere extension are now accepted as valid targets for cancer drug discovery, but the success of such strategies will still be dependent on the ability to selectively exploit a target

that may not be solely expressed in cancer cells. In addition, even if tumor-specific inhibition of telomerase becomes possible, it will probably not produce immediate cell kill; therefore, strategies employing such an approach will have to be integrated with more conventional modalities. Such conventional strategies may have reduced tumor mass to such an extent that the further cell divisions that are still likely to occur under antitelomerase therapy will not present a clinical problem. It is clear that although further knowledge has dampened the original optimism for the development of antitelomerase therapy, telomerase remains a valid target for cancer drug discovery. Agents that would inhibit it, even if not specifically in a tumor, would probably not produce toxicities normally associated with anticancer agents. Therefore, the addition of antitelomerase therapy to that of conventional therapies is likely to be beneficial.

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# Telomeres and Telomerase

## *Methods and Protocols*

Edited by

**John A. Double and Michael J. Thompson***Cancer Research Unit, University of Bradford  
Bradford, West Yorkshire, UK*

The discovery of telomerase expression in a majority of tumors and the recognition of its potential role in the aging process has dramatically raised its profile as a target for therapeutic intervention. In *Telomeres and Telomerase: Methods and Protocols*, John A. Double and Michael J. Thompson have collected a critically important series of novel and essential techniques for studying telomeres and telomerase. These readily reproducible protocols provide cutting-edge tools to identify, measure, and analyze telomeres, to determine telomerase expression at the RNA level, to determine telomerase activity, and to detect potential modifiers of this activity. The techniques for assaying telomerase activity range from standard radiological TRAP assays to nonradioactive methods, from non-PCR-based methods to techniques using real-time PCR. Written by investigators who have used the methods extensively, each protocol includes step-by-step instructions, tips on avoiding pitfalls, and invaluable notes that make all the difference to successful experimental outcomes.

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