

# Development of a quantitative luminometric hybridization assay for the determination of telomerase activity

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## Abstract

**Objectives:** To develop a quantitative luminometric hybridization assay for the determination of telomerase activity in tissue and cell extracts.

**Design and Methods:** Quantification is based on the coamplification of telomeric repeats synthesized by telomerase along with a specifically designed recombinant DNA-internal standard (DNA-IS). The DNA-IS has a similar size and the same primer recognition sites as the telomerase DNA products and differs from them only in a central 18 bp sequence. PCR products are captured on microtiter wells *via* the biotin-streptavidin system and hybridized with two distinct digoxigenin-labeled oligonucleotide probes that are designed to recognize specifically telomerase products and DNA-IS. The hybrids are quantified by a luminometric reaction using an antidigoxigenin antibody conjugated to alkaline phosphatase. The hybridization assay was validated with the MCF-7 breast carcinoma and leukemia K-562 cell lines and a synthetic telomerase product ( $R_g$ ).

**Results:** Luminescence ratios for telomerase products and DNA-IS were linearly related to the concentration of the pre-PCR product synthesized by telomerase ( $R_g$ ), in the range of 0.0005 to 10 pM. The overall reproducibility of the assay (between-run) varied between 11.3 and 15%. Application of the method in eleven breast tumors showed a great variation in the levels of telomerase enzymatic activity.

**Conclusions:** The proposed luminometric hybridization assay for the quantitative determination of telomerase enzymatic activity is highly sensitive and can be used for a large-scale prospective evaluation of clinical samples. © 2001 The Canadian Society of Clinical Chemists. All rights reserved.

**Keywords:** Telomerase activity; TRAP-PCR; Quantitative hybridization assay

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## 1. Introduction

Most higher eukaryotic cells that can divide *in vivo* cannot do so indefinitely. The process that limits the proliferative potential of cells has been termed cellular or replicative senescence. The mechanism by which cells sense the number of divisions they have completed appears to depend, at least in part, on the length of their telomeres [1]. Telomeres are specialized structures at the ends of eukaryotic chromosomes that consist of hundreds to thousands of tandem repeats of the sequence TTAGGG and appear to function in chromosome protection, positioning and replication [1,2].

Telomerase is a specific reverse transcriptase that syn-

thesizes telomeric DNA onto chromosomal ends, using a segment of its own RNA component as a template [2,3]. In vertebrate organisms, telomerase activation is the main mechanism presently known that can stabilize the loss of telomeres. Most somatic cells, regardless of their rate of proliferation undergo a steady rate of telomere loss and do not possess detectable telomerase activity. In contrast, all immortal cells examined to date show no loss of telomere length or sequence with cell division, suggesting that maintenance of telomeres is required for cells to escape from replicative senescence and proliferate indefinitely [4,5]. Recent studies in which the gene encoding human telomerase reverse transcriptase (hTERT) was transfected into normal human cells that lack hTERT, resulted in reconstitution of telomerase activity, maintenance of telomeres, extension of replicative capacity and transformation of normal cells into cancer cells [6]. The continual loss of telomeric sequence eventually reduces the production of viable cells, due to

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genomic instability since chromosomal ends are no longer protected by telomeres [7].

At present, the most commonly used method for the detection of telomerase activity employs the “telomeric repeat amplification protocol” (TRAP), in which the telomerase-synthesized DNA products are amplified by a subsequent polymerase chain reaction (PCR) [8]. This method although highly sensitive is time-consuming because involves the analysis of PCR products by polyacrylamide gel electrophoresis (PAGE) and poses potential hazards for handlers since it usually requires the use of  $^{32}\text{P}$ -labeled primers or incorporation of radioactive deoxyribonucleotides [8]. Several modifications have been described to overcome the limitations of the conventional TRAP assay. These include the use of different primers to improve the specificity of PCR amplification [9,10] and the inclusion of an internal control [10–12]. These modifications have improved the quantification of telomerase enzymatic activity but still require time-consuming post-PCR procedures [9–13]. Recently new nonisotopic assays-formats such as transcription-mediated amplification combined with hybridization protection assay [14,15], homogeneous chemiluminescent assay [16], hybridization assays that use specific fluorescent dyes that recognize double stranded DNA [17], PCR based enzyme immunoassay [18] and even the use of DNA sequencing instrumentation [19] have been reported for the quantitative determination of telomerase activity.

However many limitations concerning the quantitative determination of telomerase activity by the electrophoretic and ELISA based TRAP assays have been reported [20]. The main factor that affects the quantitative determination of telomerase activity is the difference in PCR efficiency between samples due to the presence of PCR inhibitors in tissue protein extracts [20]. Most of the quantitative methods for telomerase activity reported so far do not make use of an internal standard [13–18], while some of the methods reported for telomerase activity [10,12,19] use DNA internal standards (DNA-IS) that differ significantly in respect to size and sequence from telomerase products and are mostly suitable only for the identification of false negative results due to the presence of PCR inhibitors in tissues. However, the inclusion of a DNA-IS that has a similar size and the same recognition sites as the DNA target in the PCR mixture is an essential factor for the achievement of reliable quantitative results in PCR [21] since in this way variations in the PCR efficiency between samples are taken into account.

In most methods reported so far for the determination of telomerase activity quantification is based on the relative band intensity of telomerase PCR products on polyacrylamide gels, as estimated by scanning densitometry [9–12]. Hybridization assays can offer many advantages over the established long and tedious electrophoretic procedures for the determination of telomerase enzymatic activity, since they use solid phases and instrumentation commonly used for immunoassays in most clinical laboratories. By careful

design of the analytical methodology and specifically designed DNA-IS, hybridization assays can become quantitative, accurate and reliable [21].

In the present study we report the development of a novel sensitive and quantitative hybridization assay with lumino-metric detection for the determination of telomerase activity. This is the first report so far in which quantification of telomerase activity is based on the use of a specially designed DNA-IS that has a similar size and the same primer recognition sites as the telomerase products.

## 2. Materials and methods

### 2.1. Cell lines and tissues

The human mammary carcinoma MCF-7 and human leukemia K-562 cell-lines, that express telomerase were used as positive controls and for optimization of the assay. MCF-7 cells were grown in DMEM/Nut. Mix F12 (Gibco BRL, Life Sciences) supplemented with 10% fetal bovine serum (FBS) and 40 mg/L Gentamicin sulfate. K-562 were grown in RPMI 1640 supplemented with 10% FBS and 40 mg/L Gentamicin sulfate. The cells were quantitated by trypan blue exclusion using a hemocytometer. Tissue samples from 11 breast cancer patients admitted to Saint Savas Hospital, Athens, Greece, were collected and immediately frozen in liquid nitrogen or at  $-80\text{ }^{\circ}\text{C}$ .

### 2.2. Preparation of tissue and cell extracts

For the preparation of cell extracts  $2 \times 10^6$  cells of each cell line were washed in 500  $\mu\text{L}$  phosphate-buffered saline (PBS, 137 mM NaCl –2.7 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 1.76 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4), pelleted at 12000 rpm for 2 to 3 min at  $4\text{ }^{\circ}\text{C}$ , resuspended at 200  $\mu\text{L}$  of ice-cold CHAPS lysis buffer [10 mM Tris-HCl, pH 7.5, 1 mM ethyleneglycoltetraacetic acid (EGTA), 5 mM  $\beta$ -mercaptoethanol, 10% phenylmethylsulfonyl fluoride (PMSF), 0.5% glycerol, 100 mM  $\text{MgCl}_2$ , 0.5% CHAPS] and incubated on ice for 30 min [8]. For the preparation of tissue extracts, about 100 mg tissue samples were initially washed in 2 mL PBS, homogenized in 1000  $\mu\text{L}$  of ice-cold lysis buffer and incubated on ice for 30 min. After centrifugation at 12000 rpm for 30 min at  $4\text{ }^{\circ}\text{C}$ , the supernatants were transferred to fresh tubes and used for the quantification of telomerase activity. The protein concentrations of tissue and cell extracts were determined using the Bradford methodology [22].

### 2.3. Oligonucleotide sequences and labeling

All oligonucleotide sequences used in this study were firstly evaluated *in silico* by using the PRIMER PREMIER 4.1 software (Premier Biosoft International, Palo Alto, CA, USA) to avoid primer-dimer formation, false priming sites and formation of hairpin structures. The sequence of most of

the oligonucleotides used in this study (TS, ACX, R<sub>8</sub>, NT, TSNT) has been previously reported [8,10]. The hybridization probe Ma2 (5' 3f-AACCCTAACCCCTAACTCT-3' 3f, 18bp) was designed and used for the detection of telomerase PCR products. The hybridization probes Ma2 and NT were tailed enzymatically with Dig-11-dUTP (Roche Molecular Biochemicals, USA), by using terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals, USA), as previously described [21]. These labeled probes were used in the hybridization assay without further purification.

#### 2.4. Synthesis of DNA-IS

A DNA-IS that contains the sequences of primers TS and ACX at its 5' and 3' ends respectively and an irrelevant sequence of 18 bp (NT) between them was *in silico* designed and synthesized by PCR. Oligonucleotide TSNT that contains the sequences of primer TS and the irrelevant sequence NT [10] was used as a substrate for the PCR-mediated synthesis of DNA-IS. For this reason we used TS (18 bp) [8,10] and ACXNT (48 bp, 5'-GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC ATC GCT TCT CGG CCT TTT-3') as upstream and downstream primers respectively, for the amplification of oligo TSNT. The downstream primer ACXNT contains a 18 bp sequence at its 3' 3f-end, necessary for binding to the TSNT and a 30 bp extension (ACX) at its 5' 3f-end. PCR was performed as described above by adding 2  $\mu$ L of TSNT (100 pM) as substrate and primers TS (0.1  $\mu$ g) and ACXNT (0.1  $\mu$ g) in a total volume of 25  $\mu$ L. The resulting PCR product is the stock DNA-IS (66 bp) and was electrophoresed on a 10% polyacrylamide gel. The bands were visualized by staining with SYBR Green I (Molecular Probes, USA). A 10<sup>6</sup>-fold dilution of this stock DNA-IS solution was used as the DNA-IS working solution.

#### 2.5. Trap assay

The TRAP assay was performed as described by Kim *et al.* [10] with a slight modification in PCR conditions. Aliquots of cell and tissue extracts containing 1.5 to 14  $\mu$ g protein or different concentrations of the synthetic telomerase DNA product (R<sub>8</sub>) were added to 25  $\mu$ L reaction mixtures containing 0.1  $\mu$ g/ $\mu$ L biotinylated-TS (b-TS) primer, 0.1  $\mu$ g/ $\mu$ L ACX primer, 20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 0.1 mg/mL bovine serum albumin, 50  $\mu$ M dNTPs, 1  $\mu$ L of CHAPS lysis buffer, 1 Unit of Taq DNA polymerase (Platinum, Gibco BRL) and 1  $\mu$ L of a 10<sup>6</sup>-fold dilution of DNA-IS. Heat inactivation of both positive MCF-7 and K-562 cell extracts was performed (95 °C, 10 min) to confirm that the analytical signal was due specifically to the enzymatic activity of telomerase. All tubes were placed in a thermocycler block (Peltier Thermal Cycler, DNA engine, PTC-200, MJ Research) and incubated at 30 °C for 40 min, for telomerase-mediated extension of the b-TS substrate

primer. The mixture was further incubated for 2 min at 94 °C to inactivate telomerase and then amplified by 25 cycles of PCR at 94 °C for 30 s, 62 °C for 25 s and 72 °C for 10 min. PCR products were analyzed by electrophoresis in 0.5  $\times$  Tris-borate EDTA buffer (0.5  $\times$  TBE) on a 10% nondenaturing polyacrylamide gel and bands were visualized by staining with SYBR Green I (Molecular Probes, USA).

#### 2.6. DNA quantitation

The stock solutions of biotinylated telomerase amplification products as well as of recombinant DNA-IS were prepared by pooling several PCRs of R<sub>8</sub> and DNA-IS separately and were used for the evaluation of the sensitivity of the hybridization assay. DNA concentration of these stock solutions was determined by fluorescence spectroscopy by using SYBR Green I (Molecular Probes, USA) as a specific fluorochrome for double stranded DNA and  $\Phi$ X 174 DNA (MBI Fermentas) as a calibrator. We have optimized the DNA quantitation assay in respect to sensitivity and accuracy. Under optimized conditions, 2  $\mu$ L of various DNA standard concentrations or PCR products and 1000  $\mu$ L of SYBR Green working reagent (10000-fold dilution in 0.5 mol/L TAE buffer, pH = 8.5) were placed into a 1 mL cuvette and the fluorescence intensity was measured at  $\lambda_{exc}$  = 283 nm and  $\lambda_{em}$  = 530 nm in a fluorescence spectrophotometer (Perkin Elmer, USA). Unknown DNA concentrations were calculated by a DNA calibration graph, which was linear at a range of 50 to 500 ng/L DNA.

#### 2.7 Hybridization assay

Quantification of telomerase activity by the developed luminometric hybridization assay was performed as follows: opaque polystyrene microtiter wells (Dynatech, Microlyte 2, USA) were coated overnight at room temperature, with 50  $\mu$ L of streptavidin (1.4 mg/L) diluted in Phosphate-Buffered Saline (PBS, pH 7.4). During the whole assay the wells were washed three times after each step with wash solution [50 mM Tris (pH 7.4), 0.15 mol/L NaCl and 1 mL/L Tween-20], using a microtiter plate washer (Denley, Wellwash x4). Five  $\mu$ L of PCR products diluted 10-fold in blocking solution [10 g/L blocking reagent (Roche Molecular Biochemicals, USA) in 0.1 mol/L maleic acid and 0.15 mol/L NaCl, pH 7.5] were added in each well and incubated at room temperature with shaking for 30 min. Subsequently, 50  $\mu$ L of 0.2 mol/L NaOH were added. After a 20 min incubation the nonbiotinylated DNA strand was removed by washing the wells. Fifty  $\mu$ L of dig-labeled hybridization probe (100  $\mu$ M Ma2 or 100  $\mu$ M NT), 700-fold diluted in blocking solution, were added to each well and incubated at 42 °C for 30 min. Fifty  $\mu$ L antidigoxigenin antibody conjugated to alkaline phosphatase 750 U/L (Roche Molecular Biochemicals) diluted in blocking solution, were added per well and incubated at RT for 30 min. After this step, the

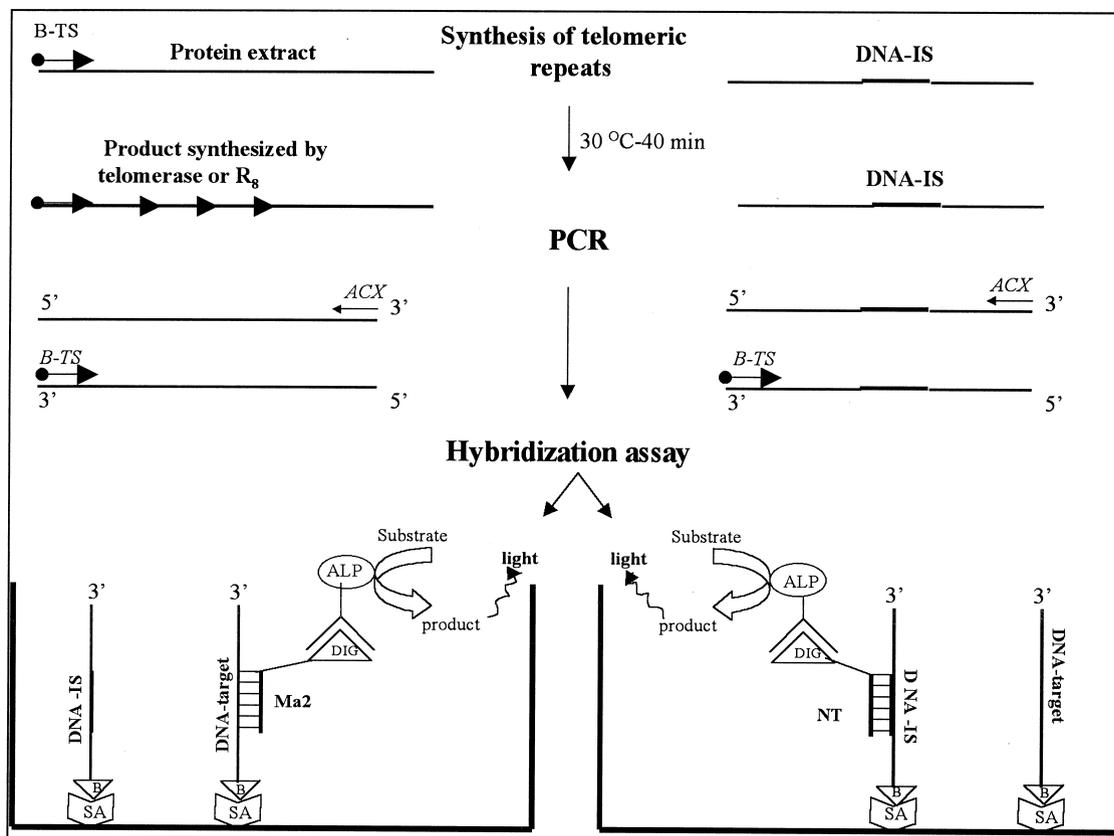


Fig. 1. Principle of the luminometric hybridization assay for the quantitative determination of telomerase activity.

wells were washed six times with wash solution and 50  $\mu$ L of the chemiluminogenic ALP-substrate LumiPhos (MEDIATORS Diagnostika, GmbH, Austria) were added to each well. The enzymatic reaction was completed after a 30 min incubation at 37 °C, in the dark. The chemiluminescence signal (expressed as Relative Luminescence Units) was measured with a Luminometer (Mediator PhL, MEDIATORS Diagnostika, GmbH, Austria).

### 3. Results

The TRAP-PCR conditions (telomerase incubation period, number of cycles, annealing temperature and PCR reaction buffer) were optimized in respect to those reported so far [10] and hot start PCR was used so that the formation of primer-dimer artifacts which is the most common problem in most TRAP based assays reported so far [8–13,20] was completely avoided. Pretreatment of the telomerase positive control (MCF-7 and K-562 cell extract) by heat inactivation abolished the synthesis of the characteristic telomeric repeat ladder of the TRAP assay, thus confirming the specificity of the reaction.

In the proposed assay, quantification of telomerase activity is based on the coamplification by PCR of DNA products synthesized by telomerase in the TRAP reaction

and a novel specifically designed DNA-IS, that has a similar size and the same primer recognition sites as the telomerase DNA products and differs from them only in a central 18 bp sequence. Calibration curves are constructed by using known amounts of synthetic telomerase products ( $R_8$ ) co-amplified with a constant amount of the internal DNA-IS.

The principle of the proposed luminometric hybridization assay for the quantitative determination of telomerase enzymatic activity is shown in Fig. 1. At first telomerase is allowed to extend its substrate TS (labeled with biotin) in the presence of the DNA-IS and then PCR follows. The biotinylated amplification products are immobilized on streptavidin coated microtiter wells and detected through hybridization to two different digoxigenin labeled probes, specific either for the synthesized telomerase products or for the DNA-IS. Hybridized amplicons are detected using antidigoxigenin antibodies conjugated to alkaline phosphatase and Lumiphos, which is a highly sensitive chemiluminogenic substrate. Hybridization assay conditions involving all steps, such as reagent concentrations, sample volume, time and temperature of incubation periods, were optimized in preliminary experiments in respect to the best signal to background ratios (S/B), sensitivity and rapidity.

The recombinant DNA-IS was initially designed in silico and finally constructed by PCR as described above. The PCR product of the DNA-IS was electrophoresed on a 10%

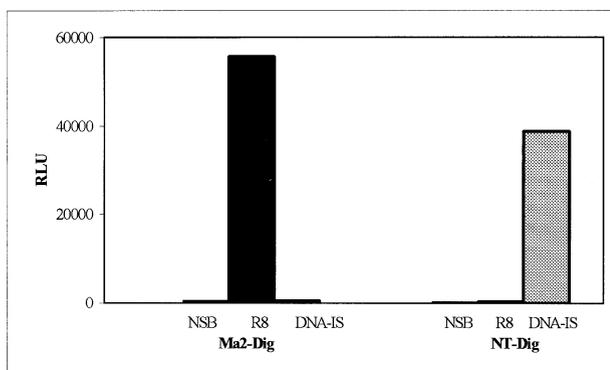


Fig. 2. Relative luminescence units (RLU) showing the specificity of hybridization probes. Probe Ma2 binds exclusively to DNA target ( $R_8$ ), whereas probe NT binds only to DNA-IS.

polyacrylamide gel and was found to have the expected size of 66 bp. Moreover to verify the presence of the new 18 bp sequence in the DNA-IS and to confirm that the amplification products from telomerase and DNA-IS were clearly distinguishable from one another the hybridization assay was performed for two samples containing the telomerase synthetic DNA product  $R_8$  and DNA-IS. The designed hybridization probes Ma2 and NT are highly specific for telomerase DNA products and DNA-IS respectively, since they can clearly distinguish them. As can be seen in Fig. 2 the luminescence signal taken from the DNA-IS with the Ma2 dig-labeled probe does not significantly differ from the background signal (non specific binding, NSB) while the luminescence signal from the DNA-target ( $R_8$ ) is high in this case. The contrary is seen when the NT-probe which is specifically designed for the DNA-IS, is used. The luminescence signals (RLU) confirm firstly that there is no cross-contamination between the DNA-target ( $R_8$ ) and DNA-IS probe and that the dig-labeled hybridization probes, Ma2 and NT, bind exclusively to the DNA target ( $R_8$ ) and DNA-IS respectively.

The sensitivity of the proposed hybridization assay was established as follows: We first prepared a stock solution of the DNA-target biotinylated amplification product by pooling several PCRs of the synthetic telomerase DNA product ( $R_8$ ). The DNA concentration of the stock was determined by fluorescence measurements using the  $\Phi X174$  DNA, (MBI, Fermentas) as DNA calibrator and the SYBR Green reagent (Molecular Probes, USA) which specifically emits fluorescence after binding to double stranded DNA. A stock solution of DNA-IS amplification product was also prepared as above. After fluorometric quantification, various dilutions of each stock solution were analyzed by the lumino-metric hybridization assay using probes Ma2 or NT. In Fig. 3 the luminescence signals (RLU) after correction for the background are plotted vs. the quantity of the amplified DNA added per well. The background is defined as the luminescence value obtained when a sample containing no amplification product (PCR negative control) was assayed. Quantities of 5 fmol for  $R_8$  and 9 fmol for DNA-IS per well

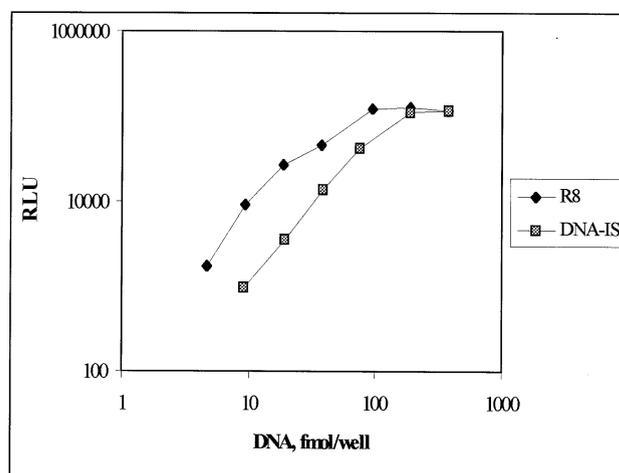


Fig. 3. Relative luminescence units (RLU) versus the concentrations of the amplified DNA ( $\blacklozenge$ : DNA target  $R_8$ ,  $\boxtimes$ : DNA-IS), showing the sensitivity of the hybridization assay ( $n = 2$ ).

were detected with signal-to-background ratios (S/B) of 6.1 and 1.8 respectively. The hybridization assay for  $R_8$  was more sensitive than the corresponding assay for the DNA-IS and this difference is probably due to folding of the captured single-stranded DNA-IS (after the NaOH step), which might interfere with the hybridization.

Calibration curves were obtained with calibrators containing known concentrations of synthetic telomerase DNA products ( $R_8$ ) in the range of 0.0001 to 10 pM and a constant amount of the DNA-IS ( $10^6$ -fold dilution of the stock). These calibrators were subjected to the TRAP-PCR amplification in parallel with the unknown samples, in which the same amount of DNA-IS was added and the amplification products were analyzed by the optimized hybridization assay. In Fig. 4, the ratio of the luminescence values obtained for  $R_8$  and DNA-IS,  $RLU_{R_8}/RLU_{DNA-IS}$ , was plotted against the pre-PCR concentrations of  $R_8$ . These data show that the

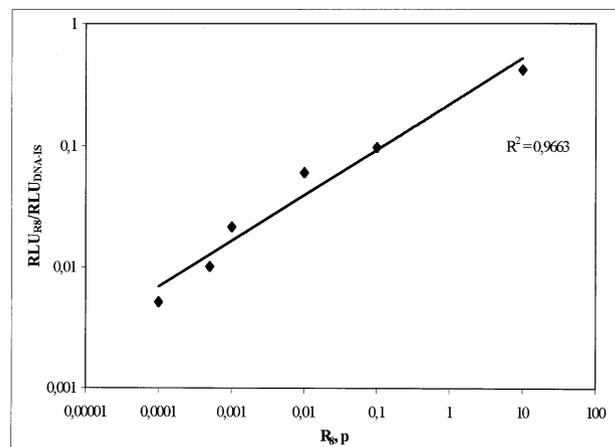


Fig. 4. Calibration curve for the quantitative determination of telomerase activity, generated by mixing known concentrations of  $R_8$  and a constant amount of the DNA-IS ( $n = 2$ ).

proposed quantitative luminometric hybridization assay for telomerase activity is linear in the range of 0.0005 to 10 pM  $R_8$ . Within-run reproducibility of the hybridization assay was tested by analyzing six times each of three different concentrations of  $R_8$  (0.001, 0.01 and 10 pM) subjected to PCR and the corresponding CVs were found to be 8.4, 6.5 and 6.9% respectively. Between-run reproducibility of the method was assayed by preparing four different samples for each of three different concentrations of  $R_8$  (0.001, 0.01, and 10 pM) and subjecting them to the whole analytical procedure and the CVs were found to be 11.6, 11.3 and 15%, respectively. The detection limit was found to be 0.1 fM for  $R_8$  (pre-PCR concentration), since this concentration could be distinguished from the background signal with a signal to background ratio of 2.

For the evaluation of the proposed assay we performed a comparison study by analyzing twenty six breast tumor protein extracts with our method and the standard electrophoretic TRAP assay [8]. Nineteen of these samples were also analyzed by a commercially available telomerase PCR-ELISA Kit (Telo TTAGGG Telomerase PCR-ELISA, Roche, CmbH, Germany). Comparison of our results with those obtained with the TRAP assay showed that seventeen samples were found positive and five samples found negative by both methods, while four samples that were found negative by the conventional TRAP assay gave high relative luminescence units (RLU > 1000) in respect to the background (RLU ~ 300) when analyzed by the proposed assay. Statistical analysis of these results by the Mc Nemar (raw data) test indicated that the differences between the proposed assay and the TRAP assay were not statistically significant ( $n = 26$ ,  $p > 0.1$ ). Comparison of our results with those obtained with the commercially available telomerase PCR-ELISA Kit showed that ten samples were found positive and five samples found negative by both methods, while four samples that were found negative by the conventional PCR-ELISA Kit were found positive by the proposed assay. Statistical analysis of these results by the Mc Nemar (raw data) test indicated that the differences between the proposed assay and the PCR-ELISA Kit were not statistically significant ( $n = 19$ ,  $p > 0.1$ ).

We believe that the discrepancy concerning the samples found positive by our method and negative by the other two methodologies could be explained by the higher sensitivity of chemiluminescence detection against absorbance and band intensity readings in polyacrylamide gels. This was also confirmed by the fact that there were no samples found negative by our method and positive by the standard TRAP assay and the commercially available PCR-ELISA Kit. The relative luminescence units and absorbance values taken for the nineteen breast tumor protein extracts analyzed by the proposed assay and the PCR-ELISA Kit are shown in Fig. 5. The samples are divided into two groups, positive ( $n = 10$ ) and negative ( $n = 9$ ) according to the standard electrophoretic TRAP assay results. As can be seen in Fig. 5 there

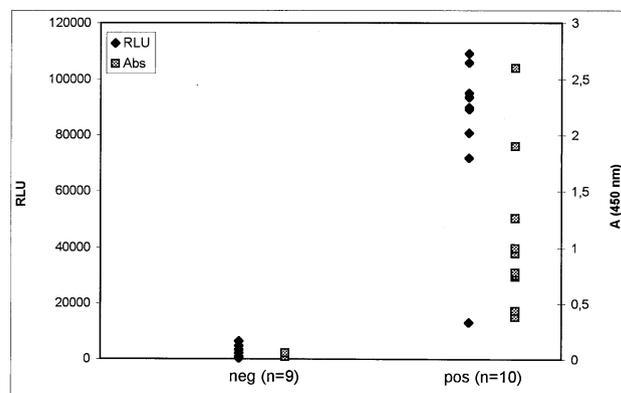


Fig. 5. Relative luminescence units (RLU) and absorbance values for 19 breast tumor tissues analyzed for telomerase activity by: (◆) the proposed luminometric hybridization assay, (◻) a commercially available telomerase PCR-ELISA Kit. Samples are divided into groups (positive and negative) according to the results obtained by the standard electrophoretic TRAP assay.

was a good correlation between the results obtained by these three different methods.

The method was successfully applied for the quantitative determination of telomerase activity in two cancer cell lines (K-562 and MCF-7), Peripheral Blood Lymphocytes (PBLs) and eleven breast tumors (Table 1). The specific enzymatic activity of telomerase was expressed as the  $\mu$ moles of DNA telomerase products (as quantitated by the calibration curve) produced per min, and normalized in respect to the protein concentration ( $\mu$ g) of each sample. As can be seen in Table 1 both cancer cell lines were found positive for telomerase activity while as expected, specific activity of telomerase was not detected in peripheral blood leukocytes (PBLs). The K-562 cell line was found negative for telomerase activity after heat inactivation thus confirming that the signal obtained was only due to the enzymatic activity of telomerase. The mean quantity of protein added per TRAP-PCR was 6.6  $\mu$ g (range 1.5–14.0  $\mu$ g). As can be seen in Table 1 in the case of breast tissue #4, the addition of the DNA-IS helps in the rapid identification of a false negative result due to the presence of PCR-inhibitors in this tissue extract. A significant variation in the levels of telomerase specific enzymatic activity in the eleven breast tumors was observed.

#### 4. Discussion

There are two major problems with any conventional PCR-based assay for the quantitative determination of telomerase activity. First, any combination of upstream and downstream PCR primers for repetitive sequences are expected to anneal efficiently to each other and form primer dimers as already reported [8–13]. Second, these primers can anneal with any repeat unit within the telomerase product resulting in the creation of DNA products differing by 6

Table 1  
Quantitative determination of telomerase activity in cell lines and tissue protein extracts

Sample	Synthetic telomerase product ( $R_8$ ), pmol/L <sup>a</sup>	Total protein, $\mu\text{g}$	Enzymatic activity: $R_8(\mu\text{mol})/\mu\text{g prot./min} (\times 10^{15})$
Cell lines			
MCF-7	0.039	2.1	0.46
K-562	0.018	1.6	0.28
K-562	n.d. <sup>b</sup>	1.6	n.d. <sup>b</sup>
(Heat inact.)			
PBLs	n.d. <sup>b</sup>	1.6	n.d. <sup>b</sup>
Breast tumors			
1	0.17	1.5	2.8
2	0.012	8.8	0.34
3	0.10	4.2	0.59
4	n.d. <sup>c</sup>	13	n.d. <sup>c</sup>
5	n.d. <sup>b</sup>	2.6	n.d. <sup>b</sup>
6	n.d. <sup>b</sup>	3.9	n.d. <sup>b</sup>
7	1.7	14	3.03
8	0.007	10	0.018
9	2.1	6.7	7.83
10	0.003	1.9	0.039
11	$\gg 10$	5.6	$\gg 44.6$

<sup>a</sup> as quantitated by the calibration curve

<sup>b</sup> not detected (n.d)

<sup>c</sup> not detected due to the presence of PCR inhibitors in the sample

PBLs: peripheral blood lymphocytes

bp during amplification and thus further complicating an absolute quantitative analysis of telomerase activity [10,12,13]. The most reliable approach to quantitative PCR is to amplify, in the same reaction tube, the target DNA and a known amount of a DNA-IS. Ideally, the DNA-IS should have the same sequence as the target itself so that they can be both amplified with the same primers. Moreover, the DNA-IS should differ in a small sequence in respect to the DNA target to distinguish the DNA target from the DNA-IS amplification products through specifically designed DNA probes [21]. However most methods reported so far for the quantitative determination of telomerase activity based on a hybridization assay approach [14–18] use DNA-IS that differ significantly in respect to size and sequence from telomerase products.

In the proposed assay quantification of telomerase specific activity is based on the use of a specially designed DNA-IS that has a similar size and the same primer recognition sites as telomerase synthesized DNA products (only differing from them in a central 18 bp sequence). The use of this DNA-IS is the main advantage of the proposed hybridization assay over the other quantitative assays reported so far [13–18], since in this way the ratios of the luminescence signals obtained for unknown samples and DNA-IS reflect the initial ratios of their concentrations in the starting mixture [21]. Thus, any variations in the efficiency of PCR affect both samples equally. Moreover the addition of DNA-IS helps in the rapid identification of false negative results due to the presence of PCR-inhibitors in tissue protein extracts.

The proposed hybridization assay is performed in a sim-

ple way in microtiter wells with instrumentation commonly used for ELISA assays in many clinical laboratories. In this way it allows simultaneous detection of large number of samples and is more practical to perform than analysis based on gel electrophoresis [9–12]. Luminometric detection offers high sensitivity and easy quantification since results are automatically expressed in RLU by the luminometer.

The DNA-target (PCR products of telomerase or synthetic telomerase DNA product  $R_8$ ) and DNA-IS are clearly distinguishable from one another through the use of specific DNA probes as confirmed by the hybridization assay. Luminescence ratios for telomerase products and DNA-IS were linearly related to the concentration of the pre-PCR product synthesized by telomerase ( $R_8$ ), in the range of 0.0005 to 10 pM. The overall reproducibility of the assay (between-run) varied between 11.3 and 15%. These coefficients of variation are comparable to those reported [20] and are consistent with the multi-step nature of the assay. The detection limit of the proposed hybridization assay was found to be 0.1 fM for  $R_8$  and is almost equivalent to that previously reported for the conventional TRAP assay [10].

Comparison studies showed that our method from a qualitative point of view gives similar results with the standard TRAP assay that is in common use today [8] as well as with a commercially available PCR-ELISA Kit for telomerase activity. Moreover its luminometric detection offers high sensitivity and easy quantification.

The developed method can quantitate the amount of DNA products synthesized by telomerase before PCR and thus the specific activity of this enzyme can be estimated

quantitatively, since the reaction time is also under control. Application of the method in eleven breast tumors showed a great variation in the levels of telomerase enzymatic activity. We are currently applying the proposed methodology in a large number of breast tumors to evaluate the clinical significance of different levels of telomerase activity in respect to other clinicopathological variants.

In conclusion, a highly sensitive luminometric hybridization assay for the quantitative determination of telomerase enzymatic activity was developed. This is the first report so far in which quantification of telomerase activity is based on the use of a specially designed DNA-IS that has a similar size and the same primer recognition sites as the telomerase products. The method is highly specific, free of false negative results and suitable for a large-scale analysis of clinical samples.

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