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Microarray TRAP—a high-throughput assay to quantitate telomerase activity

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Abstract

Telomeric repeat amplification protocol (TRAP)—a sensitive, PCR-based assay to detect telomerase activity was quintessential to the evaluation of telomerase role in telomere maintenance, cell proliferation, tumour development, and cell immortalization. The assay, however, suffers from many limitations. The most significant are: lack of telomerase activity quantification, changes of the enzyme activity product size and/or ratio, and complex post-amplification procedures which limit the assay throughput. Here we report the development of the microarray TRAP (MTRAP) assay which combines advantages of microarray technology with a modified TRAP assay. The MTRAP was designed and optimized on rice cell suspension telomerase extract to enable telomerase specific, reliable, and linear quantification in high throughput mode, with sensitivity comparable to those of radioisotope-based TRAP assays. The MTRAP has a built-in system guaranteeing the amplification of telomerase activity products unchanged in length and/or ratio and built-in control for false negatives. Thus, our MTRAP assay provides new reliable tool for experiments requiring massive quantitation of telomerase activity.
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Telomerase is one of the key players in the telomere complex, telosome, which is indispensable for chromosome protection and integrity [1]. Telosome malfunctioning destabilizes the genome and has lethal consequences for the cell [2]. Telomerase catalyses the synthesis of telomeric repeats to the end of linear eukaryotic chromosomes. Thus, telomerase activity overcomes "the end replication problem" [3] by compensating for the gradual loss of telomere sequences with each cell division [4]. Telomerase has been identified in a number of lower and higher eukaryotes, including plants [5]. The enzyme's fundamental role in maintaining telomeres and cell proliferation in fungi [6], plants [7], and mammals [8] has been well recognized and documented. The years since telomerase discovery have revealed the enzyme roles in human neoplastic transformation [9], senescence [10], cardiac [11], and ageing disorders [12]. In particular, the discovery of telomerase activity in approximately 90% of human malignancies [13] has prompted the idea of using the enzyme activity as a universal and specific marker for cancer early detection, prognosis, and patient monitoring [14]. In addition, telomerase reactivation has been shown to be sufficient to immortalize many types of human cells [15,16] which normally undergo senescence after the Hayflick limit number of cell divisions [17].

The role of the telomeric repeat amplification protocol (TRAP) in evaluating the function of telomerase in telomere maintenance, cell proliferation, tumour development, and cell immortalization has been quintessential.

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An adaptation of the TRAP assay to plant systems has proved the importance of telomerase to plant telomere maintenance [18], and established telomerase activity patterns in the development of mono- and dicot plants [reviewed in 19]. Since TRAP introduction in 1994 [13], this sensitive PCR-based assay has been widely used for telomerase activity screening in humans and other organisms.

The TRAP assay is a two-step procedure. In the first step telomerase from tissue or cell extracts elongates an oligonucleotide primer synthesizing telomere repeats to the primer's 3′ end. In the second step telomerase activity products are amplified in a PCR and separated in polyacrylamide gel. The detection of TRAP products is based on radioisotope incorporated during PCR, isotopically labelled primer and subsequent autoradiography, or non-radioactive staining of TRAP products with SYBRGreen [20]. The original TRAP assay, however, suffered from a number of limitations. A serious restraint of the TRAP assay was changes to the size and/or ratio of telomerase activity products during PCR amplification. The introduction of two reverse primers and a highly specific PCR protocol by Szatmari and Aradi solved this problem. This modified TRAP protocol does not change the distribution of the starting telomerase products during PCR amplification [21]. Another significant restraint of the original TRAP assay was that it produced qualitative telomerase measurements only, unless densitometric analysis was performed. Various modifications were suggested to overcome this limitation. For example, a TRAP internal standard (TRAPIS) was introduced to PCR. The TRAPIS molecule, different in size from those of the telomerase extension products, was designed to be amplified by the same set of primers as the products of telomerase activity [22]. The competitive, therefore PCR allowed for better quantitation of telomerase activity but still required densitometric analysis, otherwise the results were semi-quantitative. A different approach to telomerase quantification used energy transfer primers which emitted fluorescence only upon incorporation into PCR products [23], or bioluminescence where telomerase activity was measured by evaluating the amount of inorganic pyrophosphate generated in the PCR amplification of the telomerase elongation product with use of the sensitive enzymatic luminometric inorganic pyrophosphate detection assay [24]. Finally, a real-time PCR SYBRGreen-based TRAP assay has been recently developed for rapid and reliable quantification of telomerase activity [25]. The final limitation of the original TRAP assay, and many of its modified versions, was the detection of TRAP products based on reagents hazardous to human health and the environment, and the complex post-PCR steps of TRAP assay such as PAGE, autoradiography, and densitometry, all of which result in low throughput. Some closed-tube telomerase detection systems [23–25] eliminated the problem of complex post-amplification steps and improved issues of the use of hazardous reagents and low throughput, the latter however still limited to the maximum format of a 96-well microtiter plate. An exhausting review covering the methodology of telomerase activity screening from the standard TRAP protocol, through its modified and improved versions, to transcription-mediated amplification and hybridization protection assay was recently published by Saldanha et al. [26].

Here, we report on the development of the Microarray TRAP—a technique which combines the TRAP assay with microarray technology. Our intention was to enable the quantification and processivity assessment of telomerase activity in a high throughput mode without hazardous reagents. This purpose has been achieved by the use of a modified TRAP assay [27] combined with the hybridization of TRAP products printed on a microarray slide to fluorescently labelled telomeric and internal control probes. Our microarray TRAP data on rice suspension telomerase indicated strong linear correlation of the normalized telomerase signal intensity and over two ranges of magnitude in protein quantity of the rice suspension telomerase extract ($R^2 = 0.993$, $P < 0.001$), with a sensitivity comparable to those of other TRAP assays. The presented format of the microarray TRAP developed for plant systems should be adaptable to humans with minor modifications only, holding a potential promise for the technology applications in cancer diagnosis and prognosis.

Materials and methods

Plant material

The plant material used was rice (*Oryza sativa* L.) cell suspension derived and cultured according to the published methods [27]. Cell suspension was collected three days after subculturing to the fresh medium.

TRAP reaction

Telomerase extract was prepared as described [28]. In each experiment 2 μl of extract with 2.5, 0.5, 0.1, 0.02, 0.004 or 0 μg protein was used. As a negative control 2 μl of extract with 0.5 μg protein heat denatured was applied. TRAP reaction was carried out as described [27] except when (1) reaction volume and primer concentration were halved, (2) T4g32p was omitted, (3) the number of PCR cycles of the second round was changed to 35 in the MTRAP functionality test and 27, 30, and 35 in the assay optimization, (4) TRAPIS quantity used was 100 attogram in the MTRAP functionality test and 10, 50, and 100 attogram in the assay optimization.

DNA microarray technology

DNA preparation for printing and printing. TRAP reaction products were isopropanol precipitated, suspended in 25 μl of "spotting buffer" (2× SSC, 40% DMSO, and 0.02% *N*-lauroylsarkosyl), and printed in triplicate onto microscopic glass slides (*Menzel*) in GMS arrayer (*Genetic MicroSystems*).

Slides processing. To denature DNA and bind it to the slide surface, slides were baked at 80 °C for 3 h, incubated at 95 °C water bath for 2 min and submerged in 95% EtOH for few seconds.

Probe labelling. Probes for hybridization were labelled with fluorescent Cy dyes (*Amersham*). Approximately 45 cloned plant telomeric repeats (45 full and two partial repeats, 320 bp in total) were used as telomeric probe. pCR2.1-TOPO vector (*Invitrogen*) was used as probe hybridizing to TRAPIS (TOPO probe). The telomeric probe was labelled with CyDye3 in three ways: (I) using random decamer method and "*MBI Fermentas DecaLable Kit*" (*Fermentas*), (II) by elongation of telomeric primer TEL (5′-AAACCCTAAACCCTAAACCCTAAA CCC-3′) with Klenow fragment using ~45 cloned plant telomeric repeats as template, and (III) by elongation of the above TEL primer in PCR using the same template as in the method (II). The detailed description of the labelling methods is provided in the Supplementary material 1.

The TOPO probe was labelled with Cy5 with "*MBI Fermentas DecaLable Kit*" (*Fermentas*) and procedure described for (I) in Supplementary material 1.

Slides hybridization, washing, and detection. Slides were hybridized with hybridization mixture (30 μl) containing: *ExpressHyb* hybridization buffer (*Clontech*), 1 μg μl^{-1} DNA herring sperm (*Invitrogen*), the whole labelled telomeric probe obtained from the given labelling method, and 1/2 of TOPO probe quantity obtained from the labelling reaction. Hybridization was carried out at 65 °C overnight. After hybridization slides were washed at RT in: 1× SSC, 0.1% SDS for 5 min, 1× SSC for 5 min, 0.2× SSC for 2 min, and 0.02× SSC for 20 s. Hybridization signal was detected in GMS 418 Scanner (*Genetic MicroSystems*) reading fluorescence intensities for Cy3 and Cy5 channels separately. Pixel values in the resulting TIFF images depended on scanned hybridization fluorescence intensities for the given sample.

Analysis of generated data. Data generated from scanned TRAP microarrays were analysed in "*ScanAnalyze* " software (version 2.5, Stanford University).

Mathematical and statistical data analysis. In order to normalize data and calculate standardized telomerase activity (STA) for each TRAP sample, the following calculations were done:

(i) subtraction of background hybridization fluorescence from hybridization fluorescence intensity values for each of the Cy3 and Cy5 channels separately resulting in respective net values;
(ii) median of net values for all nine replications of given sample (see "Data analysis" in "Results and Discussion"), separately for channel Cy3 and channel Cy5;
(iii) ratio of Cy3 to Cy5 from their respective final median values. This ratio was called STA.

Calculated STA values were shown in logarithmic scale.

Linear regression for STA values and corresponding protein quantities was assessed with the "least squares" method and the coefficient of determination (R^2) was calculated to evaluate strength of correlation. Significance of the results was based on ANOVA and *t* test, requiring $P < 0.05$ for statistical significance.

Results and discussion

Design of microarray TRAP assay

In order to achieve a fully quantitative, high throughput assay measuring only the true products of telomerase activity without hazardous reagents, a modified TRAP assay [27] has been combined with DNA microarray technology resulting in an assay called Microarray TRAP (MTRAP). The MTRAP assay consists of two parts (Fig. 1). The first part follows the modified TRAP assay described in detail elsewhere [27]. The introduced modifications ensure that the number of telomeric repeats added by telomerase does not change during the PCR step of TRAP assay, thereby reflecting the processivity of the enzyme and allowing for quantification of telomerase activity in the assay MTRAP second part. The assay second part utilizes DNA microarray technology to detect and analyse TRAP products. Precipitated TRAP products are suspended in "spotting buffer" and printed onto microscopic glass slides, each sample in the required number of replicates. After DNA denaturation and binding to the slide surface, the slides are hybridized to the probes labelled with fluorescent dyes—green Cy3 for the telomeric probe hybridization to the amplified telomerase activity products, and red Cy5 for the TOPO probe hybridization to the products of TRAPIS amplification. Following hybridization the slides are washed in the buffers with increasing stringency to remove unbound probes and dried. Resulting microarrays are scanned and obtained images are analysed in *ScanAnalyse* software.

Data analysis

In order to obtain statistically important results and to minimize any impact of technical problems on the results quality, three replications of a given sample with three replications of sample printing (bringing the total number of replications to nine) were used in each of the described experiments. The data obtained for a given sample in *ScanAnalyse* were normalized by subtracting the background values from the hybridization intensity values in each channel, and subsequent calculation of the median for all net replication values. The normalized data were used to calculate the standardized telomerase activity (STA) according to the formula:

$$\mathrm{STA} = \frac{M_{\mathrm{med}}\Delta \mathrm{TR}_{N=9}}{M_{\mathrm{med}}\Delta \mathrm{TOPO}_{N=9}},$$

where ΔTR is the net value of telomeric probe hybridization intensity to amplified telomerase activity products, ΔTOPO is the net value of TOPO probe hybridization intensity to products of TRAPIS amplification, M_{med} is the median, and N is the replication number.

Microarray TRAP assay functionality test

The functionality test of the Microarray TRAP assay was performed using rice suspension telomerase extract which had high telomerase activity in our previous experiments [27]. The test was designed to quantitatively assess telomerase activity from rice suspension telome-

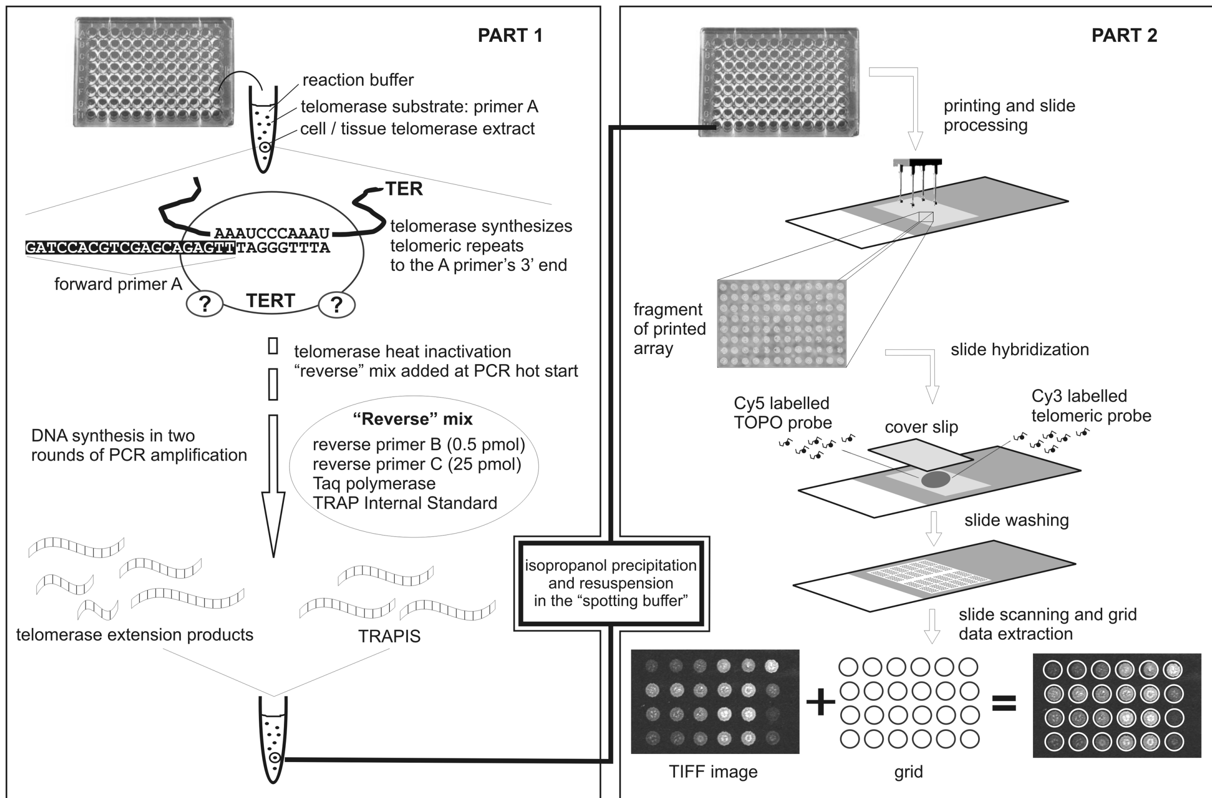


Fig. 1. A scheme of microarray TRAP assay. Part 1 follows modified TRAP assay: telomerase from cell/tissue extracts elongates substrate—primer A—synthesizing telomeric repeats to the primer's 3′ end. Primer A serves as a forward primer in a subsequent PCR amplification. After telomerase heat inactivation "reverse" mix is added at the PCR hot start. The sequence of the reverse primer B has been designed to ensure its annealing to the telomeric repeats added by telomerase, tagging telomerase extension products with the sequence of the second reverse primer C, and disabling an amplification of products of primer B slippage on telomeric repeats. After the first two cycles of amplification the remaining PCR cycling conditions are changed to allow the second reverse primer C to take over the amplification. The primer C is added in large excess in comparison to the primer B [21]. TRAP internal standard (TRAPIS) has been constructed to be amplified with the primers A and C to allow for telomerase activity quantification through competitive PCR [27] in subsequent steps of the MTRAP assay. In part 2 the TRAP products are printed on microscopic glass slides. Processed slides are hybridized to fluorescently labelled telomeric (CyDye3) and TOPO (CyDye5) probes. Following washing the slides are scanned to detect fluorescent intensities of hybridization. The grids are manually put on the resulting images to define areas of data extraction. Standardized Telomerase Activity is calculated from the extracted data of hybridization results of amplified telomerase extension products and products of TRAPIS amplification.

rase extracts of linearly decreasing protein quantity. A TRAP assay was conducted using extracts with 2.5, 0.5, 0.1, 0.02, 0.004, and 0 μg proteins in 25 μl reaction, 35 PCR cycles of the second round of amplification, and 100 attogram TRAPIS. In the second part of Microarray TRAP three methods of telomeric probe labelling were applied: (I) labelling of double-stranded DNA using the "random decamers" method and Klenow enzyme, (II) labelling of single-stranded DNA by Klenow elongation of TEL primer, and (III) single stranded DNA elongation of TEL primer in PCR. The (II) and (III) methods were used to check whether labelling of both strands of telomeric probe had the potential to create a background problem. This was expected as a result of hybridization of one of the reverse primers to the complementary strand of the labelled telomeric probe in the samples lacking telomerase activity. Results of spot blot analysis of TRAP products in experiment design as presented above, hybridized to double-stranded telomeric probe DIG labelled with the use of method (I), indicated that the background problem could be the case. Weak hybridization to negative controls (buffer without telomerase extract and heat denatured extract with 0.5 μg protein) was observed in addition to the expected decrease in hybridization intensity with decreasing protein quantity in the extracts (data not presented).

The influence upon Microarray TRAP linearity of the microchip fluorescence intensity number of readings in the scanner was tested in parallel to three labelling methods. The experiment was carried out three times with results as shown in Fig. 2. These results seem to indicate proper functionality of our Microarray TRAP assay. The logarithmic charts presented in Figs. 2A1, B1, and C1 revealed protein quantity-dependent standardized telomerase activity (STA) values, with STA values for a negative control (lysis buffer) below those for the extract with the lowest protein quantity of 0.004 μg. The STA values and corresponding protein quantities

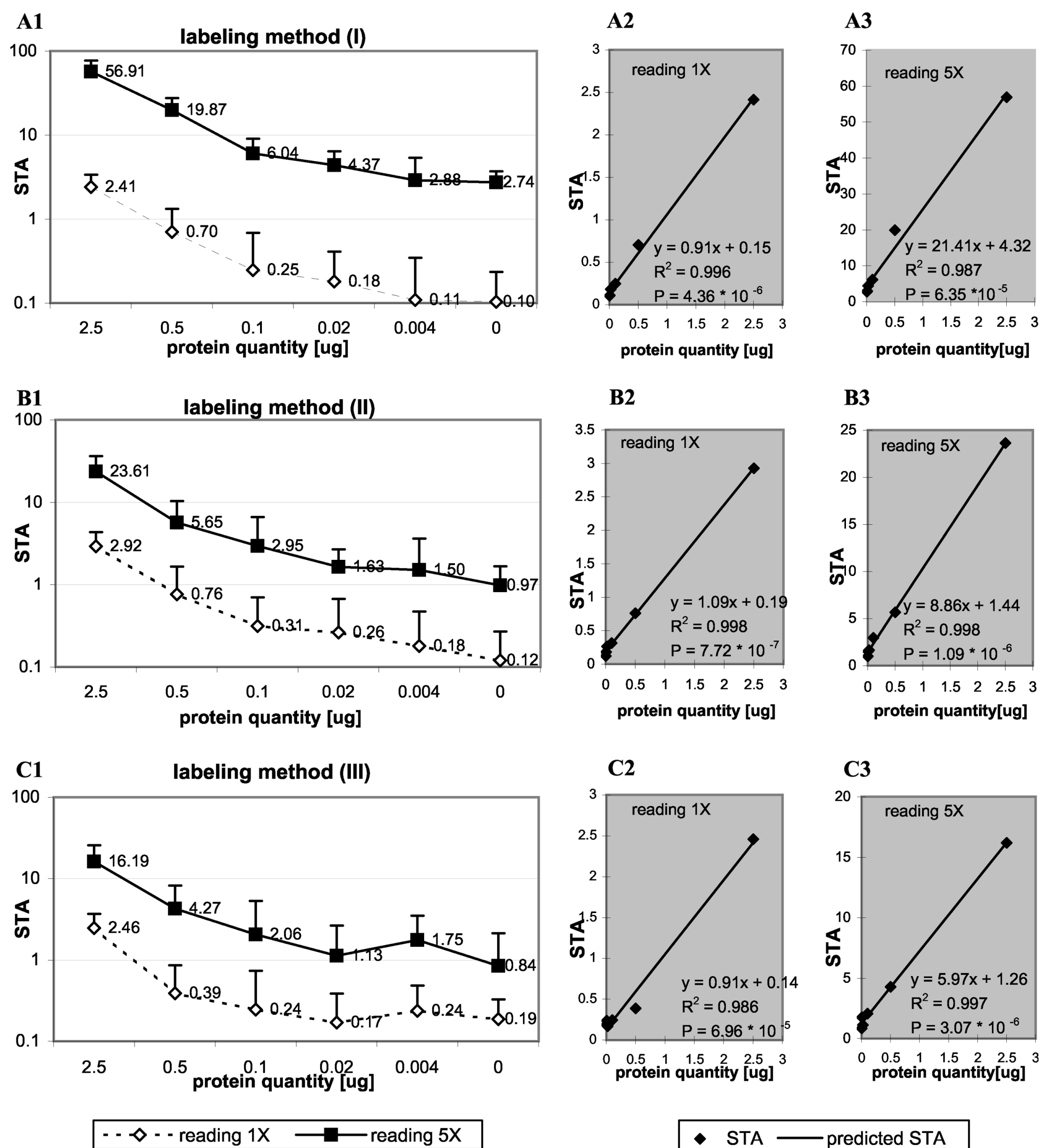


Fig. 2. Functionality test of microarray TRAP assay. Rice suspension telomerase extracts with linearly decreasing protein quantities were used to quantitate corresponding telomerase activity. Three labelling methods of telomeric probe were tested: (I) labelling of double stranded DNA using "random decamers" method and Klenow enzyme, (II) labelling of single stranded DNA by Klenow elongation of TEL primer, (III) single stranded DNA elongation of the TEL primer in PCR. In the A1, B1, and C1, the numbers above the plot represent standardized telomerase activity (STA) values, while their standard deviation values are represented by error bars. (A1) Logarithmic chart of STA values dependency on protein quantities for labelling method (I), (A2) Linear regression of STA and protein quantity for labelling method (I) and reading 1×, (A3) Linear regression of STA and protein quantity for labelling method (I) and reading 5×, (B1) The same chart as in panel (A1) but for labelling method (II), (B2) Linear regression of STA and protein quantity for labelling method (II) and reading 1×, (B3) Linear regression of STA and protein quantity for labelling method (II) and reading 5×, (C1) The same chart as in panels (A1) and (B1) but for labelling method (III), (C2) Linear regression of STA and protein quantity for labelling method (III) and reading 1×, and (C3) Linear regression of STA and protein quantity for labelling method (III) and reading 5×.

showed strong linear correlation: the R^2 coefficients for corresponding models of linear regression had high values in the range of $0.986 < R^2 < 0.998$ (Figs. 2A2, A3, B2, B3, C2, and C3). Thus, the quantitative relations between amplified telomerase activity products and products of TRAPIS amplification, obtained in PCR, were sustained during the processes of microchip preparation and hybridization. Detection of fluorescence intensity of TRAP product hybridization in the scanning of microchip and analysis of gained data translated these TRAP products' quantitative relations to numbers without losing good linear correlation between STA and protein quantities in telomerase extracts in two ranges of magnitude of protein quantities.

The Microarray TRAP assay detected signal from a sample with 4 ng proteins, thus indicating sensitivity comparable to that of the traditional TRAP assay using radioisotopes for the detection of products [13,28,29].

As anticipated the accuracy of MTRAP assay decreased with decreasing protein quantity in the sample and was the lowest for 4 ng of protein. However, distinct differences in STA values between samples with 100 and 20 ng of protein and detectable between 20 and 4 ng of protein in the samples indicate good accuracy of the MTRAP assay (Figs. 2A1, B1, and C1).

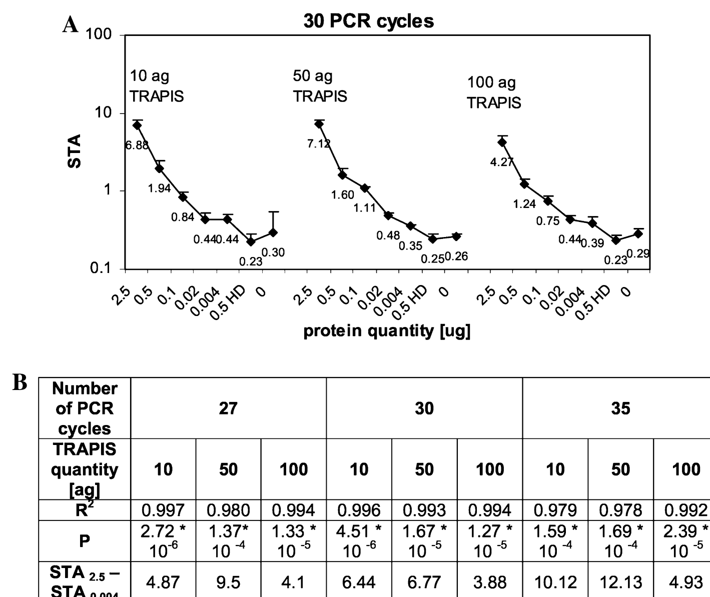
As might be expected, fivefold readings of the microchip in the scanner resulted in an increase in STA values (Figs. 2A1, B1, and C1); however, the R^2 coefficient values remained largely unaffected (Figs. 2A2, A3, B2, B3, C2 and C3). Therefore, a single reading of the microchip was considered sufficient in subsequent experiments.

No significant differences in the R^2 coefficient values for three tested labelling methods were shown (Figs. 2A2, A3, B2, B3, C2, and C3), indicating that background hybridization expected from spot blot analysis results did not distort the linear correlation between STA values and corresponding protein quantities. Similarly, no significant differences in the dynamics of the STA values were noted between the three labelling methods tested. Differences in STA values between the highest and the lowest protein quantity were 2.30, 2.74, and 2.22, respectively for (I), (II), and (III) labelling methods (Figs. 2A1, B1, and C1). Based on these results, the labelling of double-stranded DNA of the telomeric probe using the "random decamers" method with Klenow enzyme (I) was chosen as the simplest and standard labelling method for the subsequent experiments.

Further optimization of microarray TRAP assay

To further optimize the assay to improve the dynamics of the STA values, the number of second round PCR cycles was decreased to 27 and 30, as well as 10 and 50 attogram of TRAPIS were used. The rice suspension telomerase extract and quantities were the same as those used for the assay functionality test. The set of telomerase extracts was complemented with heat denatured telomerase extract of 0.5 μg protein as an additional negative control. The experiment was carried out in such a way that the extract with each protein quantity was subjected to amplification in each PCR cycle number and each quantity of TRAPIS. Apart from the new reaction parameters (27 and 30 PCR cycles and 10 and 50 attogram of TRAPIS), the parameters used for the functionality test (35 PCR cycles and 100 attogram) were sustained. The experiment was done twice with the results for 30 PCR cycles shown in Fig. 3. The results for 27 and 35 PCR cycles are presented in Supplementary material 2.

Similar to the assay functionality test, protein quantity dependent standardized telomerase activity (STA) values were revealed for all assayed combinations. STA values for the additional negative control—heat denatured extract with 0.5 μg protein—were lower than for the extract with the lowest protein quantity of 0.004 μg in all assayed combinations (Fig. 3A and Supplementary material 2). The dynamic of STA values increased with lower number of PCR cycles and less TRAPIS quantity. The least



Number of PCR cycles	27			30			35		
TRAPIS quantity [ag]	10	50	100	10	50	100	10	50	100
R^2	0.997	0.980	0.994	0.996	0.993	0.994	0.979	0.978	0.992
P	$2.72 * 10^{-6}$	$1.37 * 10^{-4}$	$1.33 * 10^{-5}$	$4.51 * 10^{-6}$	$1.67 * 10^{-5}$	$1.27 * 10^{-5}$	$1.59 * 10^{-4}$	$1.69 * 10^{-4}$	$2.39 * 10^{-5}$
$STA_{2.5} - STA_{0.004}$	4.87	9.5	4.1	6.44	6.77	3.88	10.12	12.13	4.93

Fig. 3. Microarray TRAP assay optimization. (A) Logarithmic chart of standardized telomerase activity (STA) dependency on protein quantity in rice suspension telomerase extracts, with 10, 50, and 100 attogram TRAP internal standard (TRAPIS) and 30 PCR cycles of the second round amplification. The numbers below the plot represent standardized telomerase activity (STA) values and error bars are represented by STA standard deviation values; 0.5 HD is heat denatured extract with 0.5 μg protein; ag is attogram. (B) Table summary of R^2 coefficient values, with P statistical significance, for STA values and corresponding protein quantities in the given combination of the number of PCR cycles and TRAPIS quantity. $STA_{2.5}$–$STA_{0.004}$ states for a difference of STA values between the highest and the lowest protein quantity in telomerase extract.

difference in STA values between the highest and the lowest protein quantity was noted for 100 attogram TRAPIS in all combinations of PCR cycles (Fig. 3B). The strength of correlation between STA values and corresponding protein quantities was evaluated as values of R^2 coefficient in the range of $0.978 < R^2 < 0.997$, the closest to 1 being in 30 cycles of PCR amplification (Fig. 3B). Values of R^2 coefficient were 0.996, 0.993, and 0.994, respectively, for 10, 50, and 100 attogram TRAPIS. Therefore, the combination of 30 PCR cycles and 50 attogram TRAPIS seemed to be the best combination of TRAP parameters resulting in the highest R^2 value and the best dynamics of STA values (6.77).

Features of microarray TRAP assay

Microarray TRAP combines in one assay the advantages of the modified TRAP assay and DNA microarray technology. The first part of Microarray TRAP is based on the TRAP assay developed by Kim et al. [13] and applies modifications introduced by Szatmari and Aradi [21]: the utilization of two reverse primers and a specific PCR protocol to ensure that the number of telomeric repeats present in the original telomerase products does not change on PCR amplification. Telomerase activity quantification is achieved by introducing the TRAP Internal Standard amplified by the same set of primers as the telomerase extension products, thereby ensuring that the relative abundances of the amplified telomerase extension products and the products of TRAPIS amplification depend on the initial relative quantities of particular templates [27]. The second part of Microarray TRAP utilizes the advantages of the microarray platform, above all the quantitative analysis of hybridization signal fluorescence intensity of thousands of samples at the same time. The Microarray TRAP assay, developed as described, is characterized by the following features:

1. very good linear correlation of telomerase activity and protein quantity in telomerase extract—in the optimized conditions, R^2 coefficient value was 0.993 ($P < 0.001$) in the range of two orders of magnitude of protein quantities;
2. quantitation—quantitated telomerase activity is expressed as Standardized Telomerase Activity;
3. high throughput—the number of TRAP samples and their replicates analysed at the same time is limited only by the technical parameters of the arrayer used, i.e., the GMS 417 arrayer employed in this study allows for analysis of 1152 samples with three replicates each (3456 total), while in more advanced arrayers the number of replicates is defined by the user;
4. high sensitivity and accuracy—telomerase activity is detectable to the level of 0.004 μg protein in the assayed sample, and the differences in telomerase activity are detectable between 20 and 4 ng protein in the samples;
5. built-in system guaranteeing amplification of the true length products of telomerase activity only—telomere repeat number synthesized by telomerase does not change during subsequent PCR amplification;
6. built-in control for false negatives—lack of TRAPIS amplification will indicate the presence of potential inhibitors of *Taq* polymerase in telomerase extracts.

In summary, our Microarray TRAP assay allows for analysis of telomerase activity patterns in a number of samples limited only by the experimental requirement with the number of replications needed to achieve statistical significance, and provides fully quantitative, highly specific data in three days. The two and half days invested in preparation, hybridization, scanning of microarrays, and analysis of generated data are made up by data quantity. The data quality is guaranteed by modifications introduced to the TRAP assay, optimization of PCR reaction parameters (the number of the second round PCR amplification and quantity of TRAPIS), and the possibility of analysing the number of given sample replications required to achieve statistical significance and to minimize the impact of technical problems on the quality of results. Thus, our Microarray TRAP assay seems to be a good tool for experiments requiring reliable high throughput quantitation of telomerase activity. Since telomerase activity is associated with 90% of human cancers spanning more than 20 different types of cancers [30], the enzyme activity comprises a specific and universal marker of neoplastic transformation. Human cancer dependency and disease prognosis on telomerase activity are well known and documented [31]. With relatively small modifications, the assay developed for plants could find an application in the early diagnosis and prognosis of human cancers as well as in disease monitoring.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2004.08.109.

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