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Detection of Telomerase Activity in High Concentration of Cell Lysates Using Primer-Modified Gold Nanoparticles

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Abstract: Although the telomeric repeat amplification protocol (TRAP) has served as a powerful assay for detecting telomerase activity, its use has been significantly limited when performed directly in complex, interferant-laced samples. In this work, we report a modification of the TRAP assay that allows the detection of high-fidelity amplification of telomerase products directly from concentrated cell lysates. Briefly, we covalently attached 12 nm gold nanoparticles (AuNPs) to the telomere strand (TS) primer, which is used as a substrate for telomerase elongation. These TS-modified AuNPs significantly reduce polymerase chain reaction (PCR) artifacts (such as primer dimers) and improve the yield of amplified telomerase products relative to the traditional TRAP assay when amplification is performed in concentrated cell lysates. Specifically, because the TS-modified AuNPs eliminate most of the primer-dimer artifacts normally visible at the same position as the shortest amplified telomerase PCR product apparent on agarose gels, the AuNP-modified TRAP assay exhibits excellent sensitivity. Consequently, we observed a 10-fold increase in sensitivity for cancer cells diluted 1000-fold with somatic cells. It thus appears that the use of AuNP-modified primers significantly improves the sensitivity and specificity of the traditional TRAP assay and may be an effective method by which PCR can be performed directly in concentrated cell lysates.

1. Introduction

Telomeres, which are specific nucleotide sequences occurring at the ends of linear chromosomes, are indispensable for the maintenance of chromosome integrity during replication.[1−4] Telomerase, the ribonucleoprotein enzyme[5] that recognizes and elongates the G-rich tips of existing telomere DNA repeats, is not expressed in most healthy tissues.[4,6,7] Re-expression of telomerase is often seen, however, in immortal phenotypes such as cancer.[8] Specifically, although it is repressed in most somatic cells, telomerase is active in more than 80% of all human cancers.[8,9] This renders this enzyme an important therapeutic target and a valuable marker of malignancy and tumor progression.[10]

To detect the activity of cellular telomerase, a polymerase chain reaction (PCR)-based method known as the telomere repeat amplification protocol (TRAP)[11] has been developed as a gold-standard assay. The conventional TRAP assay is a single-tube reaction in which telomerase extracted from cells or tissue synthesizes telomeric products (telomeres) first from an exogenously added telomere strand (TS) primer. These elongated products then serve as the template for PCR amplification, which is followed by polyacrylamide gel electrophoresis (PAGE) characterization and, finally, quantification via densitometry.[11] Since the invention of the TRAP assay, various modifications to improve quantification and simplify the time-consuming post-PCR steps have been reported. Examples include combining

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the TRAP assay with a post-PCR hybridization protocol employing chemiluminescent probes,[12] fluorescent dyes,[13] energy-transfer primers,[14] or biotinylated primers[15] to measure the amount of PCR-amplified double-stranded DNA.

When combined with the above-described post-PCR modifications, the TRAP assay is perhaps the most effective assay reported to date for the detection of telomerase activity. However, the limited specificity and sensitivity of the current TRAP assay remain significant challenges and make the assay difficult to use in complex samples. For example, primer-dimer products resulting from the staggered annealing of PCR primers often plague the TRAP assay,[16] and this problem has proven to be recalcitrant even to careful primer design.[17] Additionally, the TRAP assay appears to be inhibited by poorly defined interferants present in crude, highly concentrated cell extracts and other samples,[18,19] including those taken from solid tumors (e.g., tumor biopsies[20]) and bodily fluids (e.g., colonic effluents[21−23]). In response, several methods have been developed in which these inhibitors are removed or diluted prior to the PCR via phenol/chloroform extraction,[19] biotin affinity labeling and extraction,[24] or sample dilution.[21] Alternatively, Kim and co-workers have designed a 36 base pair (bp) double-stranded internal standard that allows the operator to "correct" signal for the presence of inhibitors.[25] These modified methods, however, complicate the elegant simplicity of the underlying TRAP assay.

Here we report a modified TRAP assay that leads to significantly enhanced performance in complex samples, such as concentrated, crude cellular extracts, without significantly complicating the method. The inspiration for our approach stemmed from previous studies demonstrating that the addition of gold nanoparticles (AuNPs) improves the yield of PCR reactions and reduces nonspecific amplification artifacts. For example, several groups have shown that 12 nm citrate-capped AuNPs[26,27] or primer-modified AuNPs[28] enhance the yield of PCR products obtained with a single purified template[26−28] or genomes extracted from culture medium or whole blood.[29] To date, however, there have been no reports of AuNP-based PCR assays working directly in highly concentrated cell extracts, presumably because citrate-capped AuNPs aggregate at concentrations above 0.9 nM at the standard ionic strengths required for PCR reactions. To circumvent this problem, we have modified citrate-capped AuNPs with a PCR primer (modified TS primer). Using the conventional TRAP assay as a test bed, we have shown that such primer-modified AuNPs used at high concentrations significantly improve the specificity and sensitivity of PCR performed directly in complex, interferant-laced samples. Our AuNP-modified TRAP assay directly detects low levels of cancer-indicative telomerase activity in cancer cell extracts that have been doped with a 1000-fold higher concentration of somatic cell lysates.

2. Experimental Section

2.1. Preparation and Characterization of TS-Primer-Modified AuNPs. Citrate-capped AuNPs (12 ± 1 nm) were prepared according to literature procedures,[30] and the AuNP concentration was determined by measurement of the absorbance at 519 nm. Oligonucleotides **1** and **2** (modified TS primers) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and purified by PAGE. The sequences of modified TS primers were the following:

1:5′-HS$(CH_2)_6$TTTTTTTTTAATCCGTCGAGCAGAGTT-3′

2:5′-HS$(CH_2)_6$TTTTTTTTTTTTTTTTTAATCCGTCGAGCAGAGTT-3′

The TS-modified AuNPs were prepared as reported previously.[31] The DNA/AuNP ratio was calculated by the following method:[31] The absorbance spectra of the AuNPs before and after DNA modification were measured. Both spectra were normalized to an absorbance of 1 at the 519 nm plasmon peak of the AuNPs. The molar ratio was calculated using the known extinction coefficients of DNA strands at 260 nm and the NPs at 519 nm. The calculated loading corresponded to ∼50 TS primers per AuNP.

2.2. Preparation of Telomerase Extracts. Human breast carcinoma cell lines MDA-MB-231, MCF-7, and T47-D were obtained from American Type Culture Collection (ATCC, Manassas, VA). T47-D was cultured in RPMI-1640 medium, MDA-MB-231 in DMEM, and MCF-7 in EMEM supplemented with 10% fetal bovine serum and 1× pen/strep (Invitrogen, Carlsbad, CA). Human mammary epithelial cells (HMEC) were cultured in mammary epithelial cell medium obtained from Lonza (Walkersville, MD). After the cells were harvested with trypsin, 1 million cells were collected into microcentrifuge tubes and centrifuged at 2000 rpm for 10 min at 4 °C. Cells were washed once in phosphate buffered saline (pH 7.4), centrifuged again, and frozen at −80 °C. The cells were resuspended at a concentration of 1×10^6 cells/mL in cold 1× CHAPS lysis buffer [10 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 0.5% CHAPS (Sigma), and 10% glycerol] plus 2 mM DTT and 200 units/mL RNase inhibitor. The resuspended cells were then incubated for 30 min on ice before

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being centrifuged for 20 min. (12 000 rpm, 4 °C). After centrifugation, 160 μL of the supernatant was transferred into a fresh tube, flash-frozen, and stored at −80 °C before use.

2.3. AuNP-Modified TRAP Assay. A $TRAP_{EZE}$ telomerase detection kit (S7700) was purchased from Chemicon International, Inc. (Billerica, MA). The composition of the 25 μL PCR reaction mixture was 16 μL of PCR water, 2.5 μL of 10× TRAP reaction buffer, 0.5 μL of 50× dNTP mix, 3 μL of AuNP−TS primer (TS-modified AuNPs were added to the PCR reaction system to an appropriate concentration), 0.5 μL of TRAP primer mix, 0.5 μL of Taq polymerase (5 units/μL), and 2 μL of cell extract. These tubes were then incubated in a thermocycler at 30 °C for 30 min to allow telomerase elongation. Afterward, a three-step PCR (94 °C/30 s, 62.9 °C/30 s, 72 °C/1 min) was performed for 33 cycles. The PCR products were analyzed by agarose gel electrophoresis [4.0% low-melting agarose (Fisher Scientific), 100 V for 60 min]. The gel was stained for 20 min with SYBR Green (Molecular Probes) and imaged using with a Gel Logic EDAS 290 digital imaging system (Kodak, Rochester, NY).

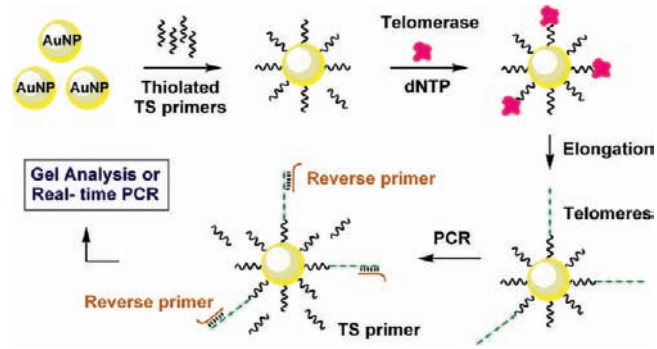
2.4. Real-Time PCR of the TRAP Assays. Real-time PCR was performed with an iQ^5 instrument (Bio-Rad, Hercules, CA). A PCR mixture containing 2.5 μL of 10× TRAP reaction buffer, 0.5 μL of 50× dNTP mix, 3 μL of TS primer (1/6 dilution) or AuNP-TS primer (TS-modified AuNPs were diluted to an appropriate concentration), 0.5 μL of TRAP primer mix, 0.5 μL of Taq polymerase (5 units/μL), and 0.15 μL of SYBR Green (1/100 dilution from a stock solution) was combined with 2 μL of cell extract and nuclease-free water to bring the total volume to 25 μL. The telomerase elongation step took place at 30 °C for 30 min, and a three-step PCR (94 °C/30 s, 62.9 °C/30 s, 72 °C/1 min) was performed for 33 cycles. Amplification results were evaluated by plotting threshold cycle (C_T) values as a function of cell number and by performing a dissociation analysis.

3. Results and Discussion

3.1. Design of the AuNP-Modified TRAP Assay. The commercial TRAP assay (Chemicon kit S7700) is a one-buffer, two-enzyme system utilizing telomerase elongation and PCR amplification. The assay involves the use of cellular telomerase to elongate exogenously added oligonucleotide primers (TS primers). The elongated TS primers serve as templates and are then amplified to detectable levels using PCR. Recently, Fan[26] and Liu[27] showed that when they are deployed in the amplification of purified single-DNA sequence, 0.2−0.8 nM citrate-capped AuNPs enhance the PCR yield and reduce nonspecific amplification of byproducts. However, the suitability of this method for direct deployment in complex samples such as crude cellular extracts has not been previously reported. To explore whether the addition of citrate-capped AuNPs would enhance the efficiency of the TRAP assay, we tested 0.2−0.8 nM citrate-capped AuNPs with the commercial telomerase detection kit (Chemicon kit S7700). However, we did not observe any enhancement in the PCR yield and specificity (data not shown). Our attempts to obtain this enhancement by performing the TRAP assay in the presence of higher concentrations of unmodified AuNPs also failed: under the employed conditions, citrate-capped AuNPs aggregated at concentrations above 0.9 nM, and the precipitate did not enhance the PCR yield (data not shown).

In contrast to unmodified AuNPs, oligonucleotide-modified AuNPs are stable in the solution, which contained high-concentration salts because of the densely packed monolayer of polyanionic DNA on their surface,[32] allowing us to raise the concentration of AuNPs in the reaction. We developed a modified TRAP assay using modified TS primers covalently attached to gold nanoparticles (TS−AuNPs) in place of the traditional TS primers. To do so, we functionalized citrate-capped AuNPs with freshly reduced, thiolated TS primers **1** (28 bases) and purified the resulting TS−AuNPs using Centricon columns (10 000 Da nominal molecular weight cutoff). The calculated surface coverage[31] of the TS−AuNPs indicated an average loading of ∼50 TS primers per AuNP. The negatively charged TS primers on the AuNPs inhibit aggregation, and thus, the TS−AuNPs remained quite soluble and separated even at the relatively high salt concentrations required for PCR. This allowed us to use the TS−AuNPs directly in the TRAP assay at concentrations of greater than 1.2 nM.

Scheme 1. Schematic Illustration of the AuNP-Modified TRAP Assay, in Which TS Primers Covalently Attached to Gold Nanoparticles Serve Both as Substrates for Telomerase-Induced Elongation and Templates for Subsequent PCR Amplification in the Same Tube



3.2. Performance of the AuNP-Modified TRAP Assay with Cancer or Somatic Cells. In the first step of the commercial TRAP reaction, telomerase adds a number of telomeric repeats (GGTTAG) onto the 3′ end of its TS oligonucleotide (18 bases in length), which acts as the substrate. In the second step, these extended products are PCR-amplified using their TS and reverse (RP) primers, generating a ladder of products with six-base increments, typically starting at 50 nucleotides (50, 56, 62, etc). When we replaced the traditional TS primers with our TS−AuNPs (modified TS primer **1** is 28 bases in length), we observed that these AuNP-attached TS primers were able to serve as both substrates for telomerase-induced elongation and templates for subsequent PCR amplification in a single tube, generating a ladder of products with six-base increments starting at 60 nucleotides (60, 66, 72, etc.) (Scheme 1).

The AuNP-modified TRAP assay showed significantly improved performance relative to that of the standard, commercially available assay. For example, using the commercial telomerase detection assay, we observed a significant 50-base primer-dimer artifact[23] when the assay was performed using extracts of somatic cells [human mammary epithelial cells (HMEC)], presumably lacking telomerase activity, as a negative control. This artifact has the same length (lane 12 in Figure S1 in the Supporting Information) as the shortest amplified telomerase product (50 bases, arising from templates consisting of four telomeric repeats added to a TS primer)[33] observed when extracts of human breast carcinoma (MCF7) cells were employed (Figure S1, lane 11), and thus, it degrades the specificity of the traditional TRAP assay.

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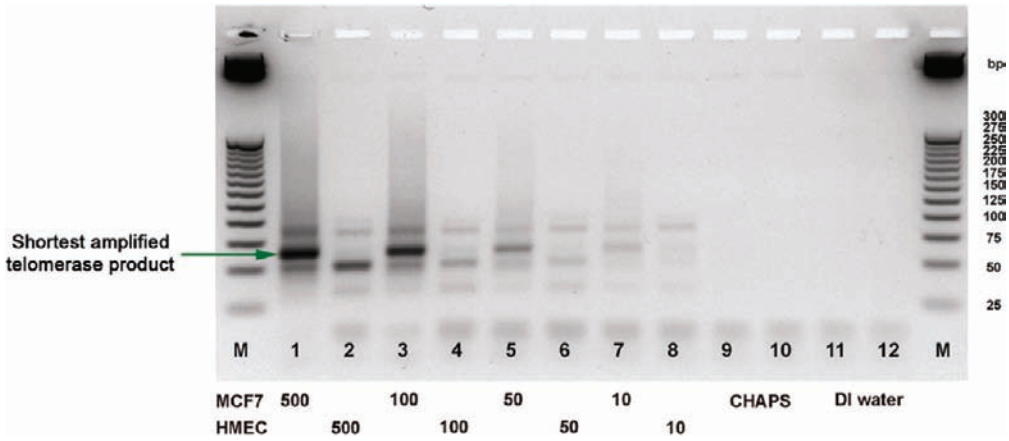

Figure 1. The AuNP-modified TRAP assay exhibits sufficient sensitivity to detect 10 cancer cells. Shown are assays performed using either telomerase-positive human breast carcinoma cells (MCF7) or telomerase-negative human mammary epithelial cells (HMEC). Samples derived from 500, 100, 50, and 10 MCF7 cells (lanes 1, 3, 5, and 7) exhibited a strong band at 60 bp indicative of telomerase activity. In contrast, samples derived from similar numbers of HMEC cells (lanes 2, 4, 6, and 8) and control samples containing only CHAPS buffer blanks (lanes 9 and 10) or distilled water (lanes 11 and 12) did not exhibit this telomerase-specific band.

In contrast, when we employed AuNP-modified TS primers in place of the traditional TS primers, we observed a highly amplified signal from the shortest telomerase product (60 bp, arising from elongation of the 28-base TS primer with four telomeric repeats) and a smear of amplified telomerase products larger than 60 bp (due to elongation by the addition of more than four repeats) when MCF-7 cancer cell extracts were tested (Figure 1, lanes 1, 3, 5, and 7). This observation is consistent with the literature, which suggests that the addition of AuNPs favors the amplification of shorter products over longer ones when PCR amplification of variable-length templates is performed.[34] We did not observe any 60 bp product when extracts of HMEC somatic cells were employed; only weak primer-dimer artifacts were clearly observed at 56, 86, and 107 bp (Figure 1, lanes 2, 4, 6, and 8). Control experiments with the AuNP-modified assay employing distilled water or CHAPS buffer blanks in place of cell extracts did not produce any observable PCR product bands (Figure 1, lanes 9, 10, 11, and 12).

To optimize the AuNP-modified TRAP assay, we tested different concentrations of TS−AuNPs in the reaction. We observed that the amplification of the 60 bp telomerase product was enhanced at TS−AuNP concentrations ranging from 0.9 to 2.5 nM (Figure S1, lanes 9, 7, 5, and 3) and strongly inhibited at TS−AuNP concentrations above 4.9 nM, where no product bands were observed (Figure S1, lanes 1 and 2). This observation is in agreement with the work of Fan et al.,[26,35] who reported that excess AuNPs inhibit PCR. This inhibition may be due to the adsorption of polymerase on the AuNPs.[34−36] The intensity of primer-dimer artifacts also varied with the initial TS−AuNP concentration. For example, the 86 and 107 bp dimer artifacts weakened or even vanished as the TS−AuNP concentration was reduced below 1.2 nM (Figure S1, lanes 4, 6, 8, and 10).

3.3. Coupling Real-Time PCR with the TRAP Assay. To further demonstrate the advantage of the AuNP-modified TRAP assay when it is performed in complex, protein-rich samples, we carried out a real-time PCR employing the fluorescence of SYBR Green to quantify the yield of amplified double-stranded PCR products. Using this approach and our modified assay, we characterized extracts of cancer cells, somatic cells, or a mixture of the two either with or without TS-modified AuNPs. As expected, the fluorescence change produced by the TS-modified AuNPs was very small relative to the change in fluorescence produced by the amplified PCR products (data not shown), which is consistent with the previous report by Fan and coworkers,[37] who noted that despite the potential quenching effects of added AuNPs, absolute quantification in real-time PCR was not obviously affected by the addition of unmodified AuNPs. This said, the addition of TS−AuNPs retarded somewhat the upturn in product formation used to monitor real-time PCR. For example, the threshold cycle (C_T) values of the extract of 100 cancer cells were 25.22 ± 0.06 when 1.2 nM TS-modified AuNPs were employed (Figure 2A) and 21.67 ± 0.04 in the traditional TRAP assay (Figure 2B). Nevertheless, when the same cancer cell extracts were doped into an extract of 10 000 normal somatic cells, the AuNP-modified TRAP assay significantly outperformed the traditional TRAP assay. Specifically, whereas the TS−AuNP-modified TRAP assay performed nearly identically in the presence (C_T = 25.81 ± 0.04) and absence (25.22 ± 0.06) of a 100-fold excess of somatic cells, the performance of the traditional TRAP assay was negatively affected by the addition of the somatic cell background (C_T changed from 21.67 ± 0.04 to 23.82 ± 0.13) (Figure 2). The poor PCR performance of the traditional TRAP assay in the presence of a high concentration of somatic cell extracts could be due to significant contamination of the PCR inhibitors from the cell extract.[29,33,38] We also noted that the PCR byproduct from CHAPS buffer blanks in the AuNP-modified TRAP assay were greatly inhibited (no C_T value was observed, as the amplification fluorescence signal did not exceed the fluorescence threshold) in comparison with the large amount of PCR byproduct produced from CHAPS buffer blanks in the traditional TRAP assay (a C_T value of 25.20 ± 0.12 was observed) (Figure 2).

In order to verify that the apparently spurious amplification observed from somatic cell samples differs from that

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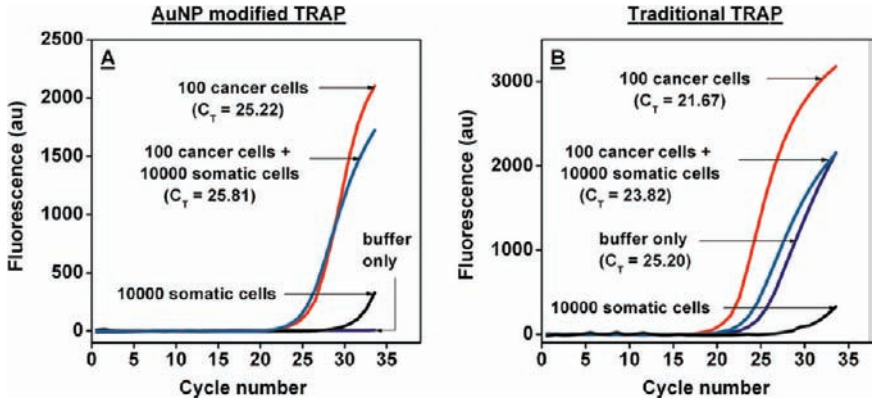

Figure 2. Real-time PCR DNA amplification curves of the extract of cancer cells only, somatic cells only, a mixture of the two, and buffer-only blanks using (A) the AuNP-modified TRAP method or the (B) the traditional TRAP assay.

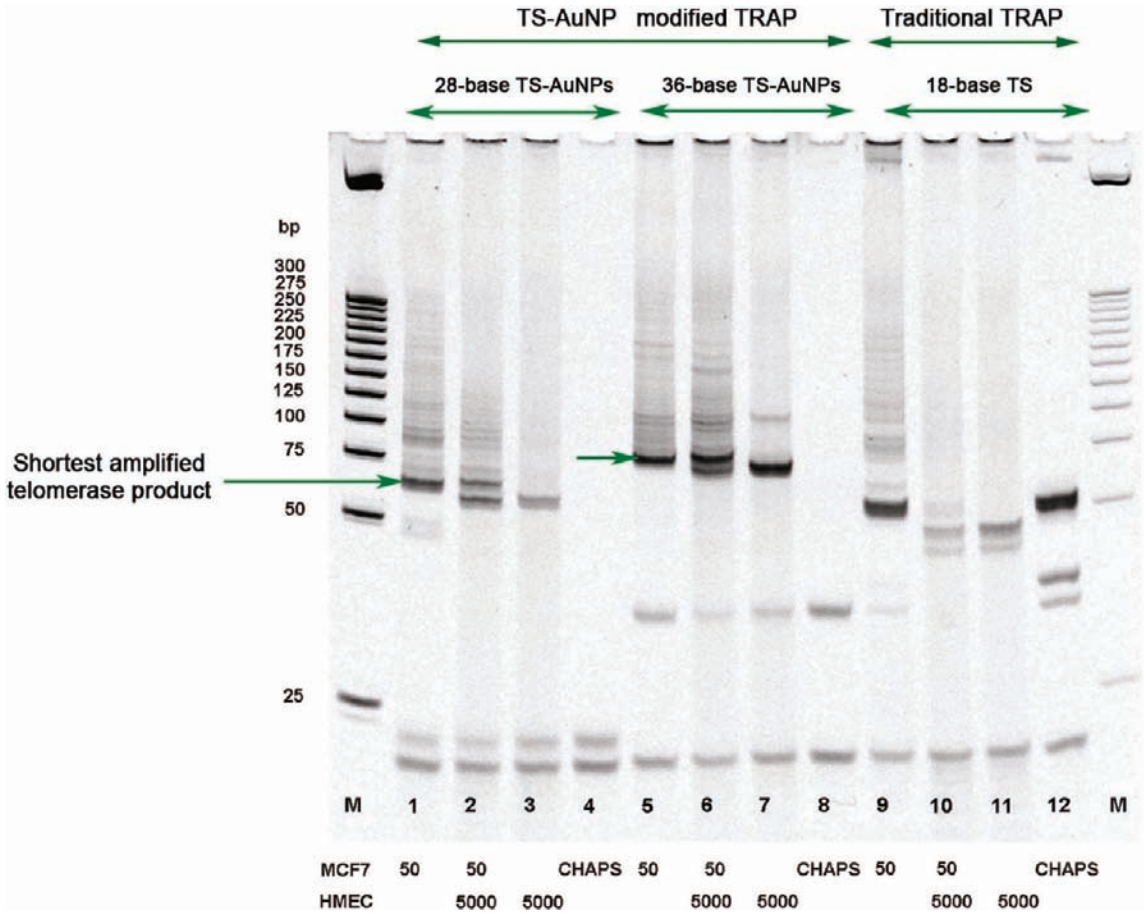

Figure 3. The AuNP-modified TRAP assay produces a six-base periodic pattern of products. Using either primer, the AuNP-modified TRAP assay readily detected the telomerase activity of 50 MCF7 cancer cells in isolation (lanes 1 and 5) or when doped in 5000 telomerase-negative HMEC cells (lanes 2 and 6), and no detectable telomerase activity was observed from 5000 HMEC cells alone (lanes 3 and 7). In contrast, with the traditional TRAP assay, only weak telomerase activity was detected from 50 MCF7 cells (lane 9), and no telomerase activity was detected from 50 MCF7 cells doped in the extract of 5000 HMEC cells (lane 10) or from 5000 HMEC cells alone (lane 11).

produced from cancer cells, we also performed a DNA dissociation analysis on our amplification products. This is an essential step when using nonspecific double-stranded DNA binding dyes such as SYBR Green in real-time PCR, as these can produce false positives due to nonspecific amplification. Melting curve analysis of the PCR products generated using the AuNP-modified TRAP assay showed a single peak at 81.5 °C for the cancer samples, peaks at 77.5 °C and 80.2 °C for somatic cells, and no peak from CHAPS buffer blanks (Figure S2 in the Supporting Information). The presence of distinct peaks for somatic and cancer cell samples indicates that different PCR products were formed from the two types of cells. For the PCR products generated via the traditional TRAP assay, we observed a single peak at 82 °C for cancer cell samples, double peaks at 77.5 and 81 °C for somatic cell samples, and a single peak at 77.5 °C for CHAPS buffer blanks (Figure S3 in the Supporting Information), indicating that the same byproduct was generated in the blank and somatic cell samples.

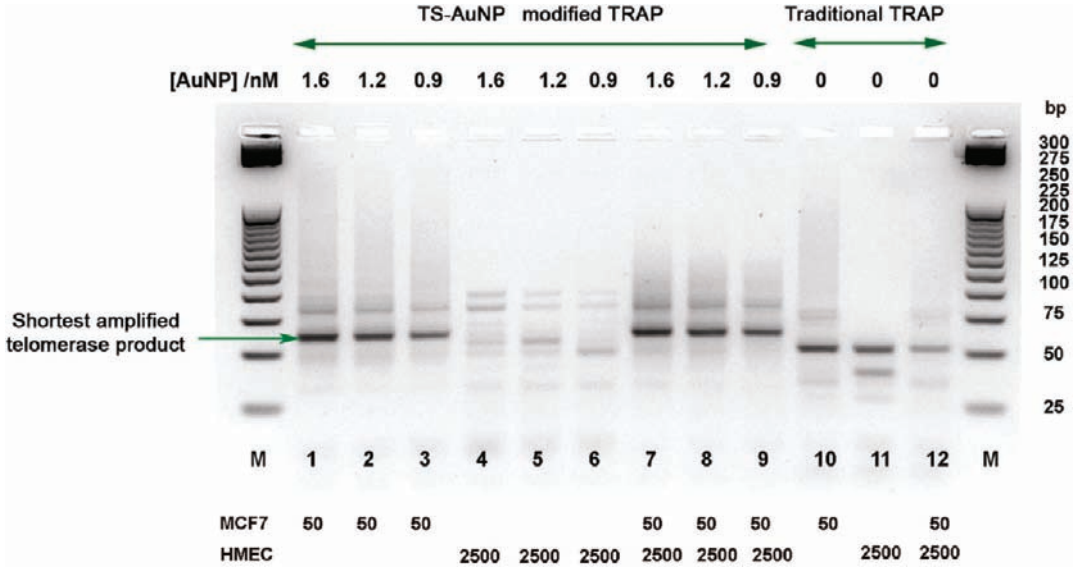

Figure 4. The selectivity of the AuNP-modified TRAP assay is significantly better than that of the traditional TRAP assay. For example, the AuNP-modified assay produced a clear telomerase product band at 60 bp from a sample of 50 cancer cells (MCF7) irrespective of whether they were in isolation (lanes 1−3) or doped in a 50-fold excess of somatic cells (HMEC) (lanes 7−9), and while some artifactual bands were seen, no telomerase-indicative band at 60 bp was observed from samples consisting only of somatic cells (lanes 4−6). In contrast, the traditional TRAP assay produced a similar pattern of bands from both cancerous and somatic cells (lanes 10−12). The triplicate lanes (lanes 1−3, 4−6, and 7−9) represent replicate measurements produced with TS−AuNPs at concentrations of (from left to right) 1.6, 1.2, and 0.9 nM.

3.4. Effect of Different TS-Primer-Modified AuNPs on TRAP Performance. When we employed agarose gel electrophoresis to monitor our products, we observed only a single clear band at 60 bp and a smear of >60 bp amplified telomerase products. When we employed higher-resolution PAGE instead, we were able to visualize the expected 6 bp ladder of products. Indeed, using AuNPs modified with 28- or 36-base telomerase TS primers, we saw the expected ladders offset by the six-base difference in the lengths of these two primers (Figure 3, lanes 1 and 5). This also held true when our AuNP-modified assay was used to detect telomerase activity in extracts of 25 cancer cells doped into 5000 somatic cells (Figure 3, lanes 1 and 2 for the 28-base TS primer and 5 and 6 for the 36-base TS primer), where somatic cell extracts produced only a 56 bp primer-dimer artifact and a smeared background (Figure 3, lanes 3 and 7). We did not observe any telomerase products in CHAPS buffer blanks (Figure 3, lanes 4 and 8), which is consistent with the obtained real-time PCR results (Figure 2). In contrast, in the traditional TRAP assay, the expected ladder of telomerase products was observed only for extracts lacking added somatic cells (Figure 3, lane 9); the addition of 5000 somatic cells almost completely inhibited the amplification of the elongated telomerase products (Figure 3, lane 10), and significant primer-dimer artifacts were obtained in the extract of 5000 somatic cells and CHAPS buffer blanks (Figure 3, lanes 11 and 12). These results further indicate that all of the cancer-related samples in our AuNP-modified TRAP assay (Figure 3, lanes 1, 2, 5, and 6) demonstrate not only the typical feature, the 6 bp periodic pattern of products, but also the significantly amplified shortest telomerase product (60 bp), allowing us to directly use the 60 bp product band on agarose to identify the cancer-related samples.

3.5. Performance of the AuNP-Modified TRAP Assay in Crude Cellular Extracts of Cancer Cells Doped with Somatic Cells. The traditional TRAP assay does not function effectively in protein-rich clinical samples such as extracts containing a mixture of telomerase-positive cells within a large background of telomerase-negative cells (i.e., high levels of contaminating proteins from the somatic cells, which dilute the concentration of telomerase) or a very small number of telomerase-positive cells in a large volume of fluid (e.g., the detection of bladder cancer using cells sedimented from urine).[22,38] For example, the traditional TRAP assay exhibits very poor specificity for the detection of the extract of small numbers of cancer cells doped in a large excess of somatic cells: we observed a clear 50 bp product band and a faint smear band from the extract of 50 cancer cells (Figure 4, lane 10) as well as a 50 bp artifact byproduct also formed in the presence of an extract of somatic cells (Figure 4, lane 11). With further dilution of the extract of 50 cancer cells with the extract of 2500 somatic cells, we observed at least a 6-fold inhibition of the PCR product yield (Figure 4, lane 12).

In contrast, the AuNP-modified TRAP assay is quite selective and readily detects small numbers of cancer cells against a 2 orders of magnitude excess of telomerase-negative somatic cells. Using the same samples tested above with the traditional TRAP assay, we observed amplified products (at 60 bp and above as a heavy smear) from an extract of 50 cancer cells in isolation (Figure 4, lanes 1−3) or when doped in a 50-fold excess of somatic cells (Figure 4, lanes 7−9). In contrast, extracts of somatic cells lacking telomerase produced only a few weak bands (Figure 4, lanes 4−6). Notably, a higher concentration of TS−AuNPs (∼1.2 nM) is optimal when monitoring telomerase activity in these protein-rich cell lysates.

3.6. The AuNP-Modified TRAP Assay Is Sensitive and Quantitative. The traditional TRAP assay suffered when challenged with extracts from 25 cancer cells doped in 1000 somatic cells (Figure 5, lane 7), and no difference between telomerase-positive and -negative samples was apparent when 25 cancer cells were doped in an extract of more than 2500 somatic cells (Figure 5, lanes 8, 9, 11, and 12). The AuNP-modified TRAP assay, in contrast, was significantly more selective. For example, the modified TRAP assay readily detected as few as 25 cancer cells doped in 5000 somatic cells (Figure 5, lanes 1−3); extracts

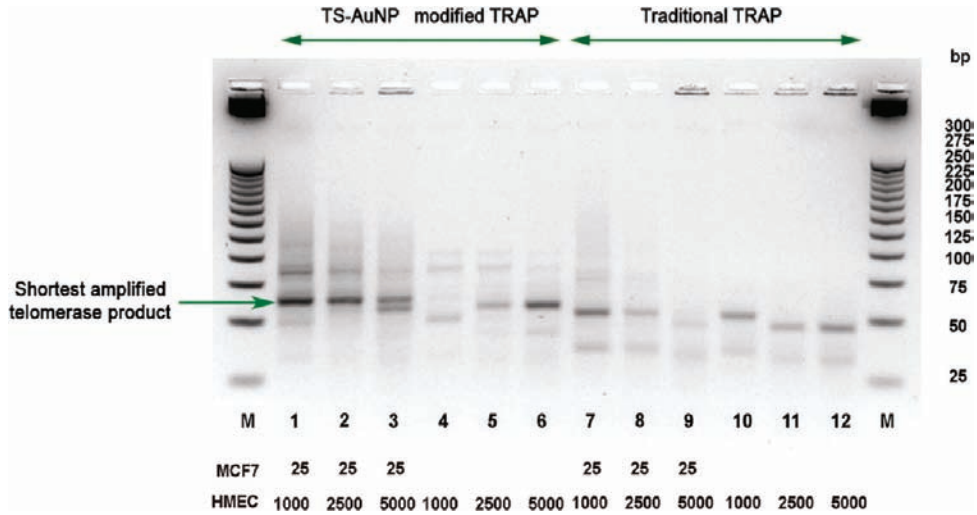

Figure 5. The AuNP-modified TRAP assay is sufficiently selective to ensure the detection of cancer cells (here MCF7 cells) even when they are diluted with a large (up to 200-fold) excess of somatic cells (here HMEC cells) (lanes 1−3). The traditional TRAP assay, in contrast, is significantly less selective (lanes 7−9).

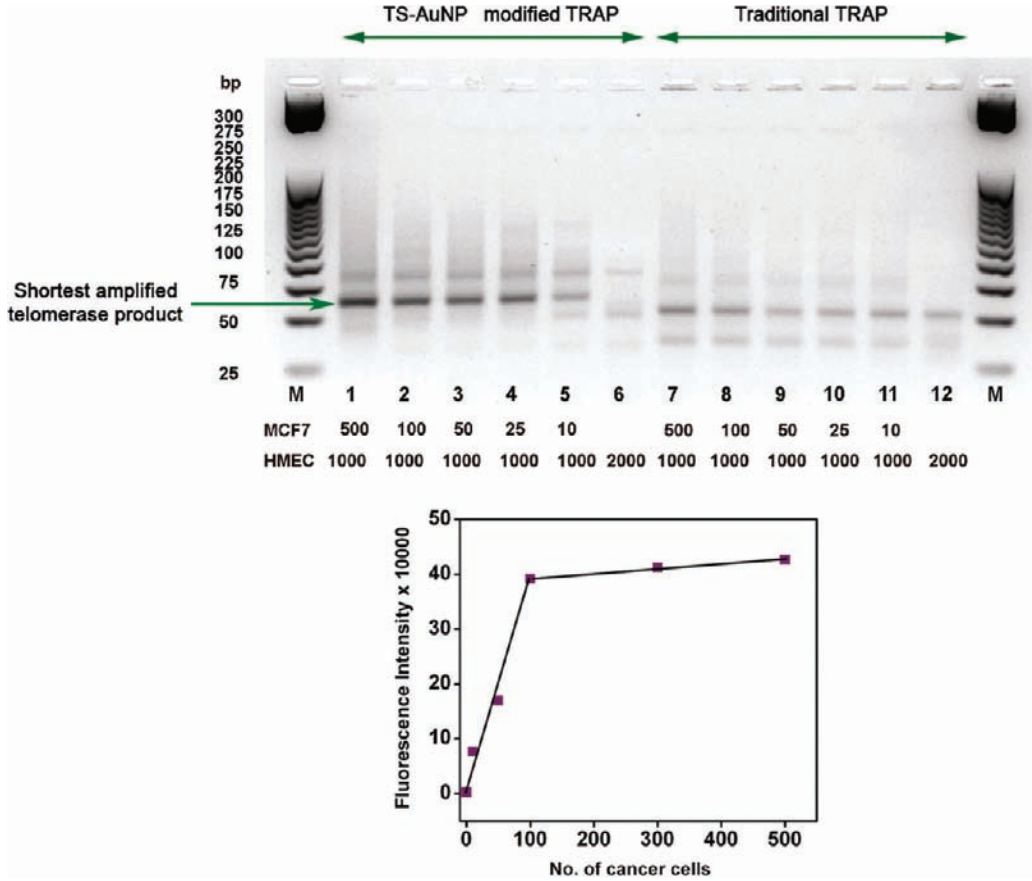

Figure 6. The yield of telomerase PCR product increases monotonically with increasing number of cancer cells in the AuNP-modified TRAP assay. For example, against a background of 1000 somatic HMEC cells, the intensity of the telomerase-indicative 60 bp band produced by the AuNP-modified assay (top panel) was closely correlated with the number of MCF7 cells in the sample (bottom panel).

from only somatic cells produced only a primer-dimer artifact at or below 60 bp (Figure 5, lanes 4−6). A heavily smeared band was also well-correlated with extract samples that included telomerase-positive cancer cells (Figure 5, lanes 1−3 and 7).

Even when deployed in complex mixtures, the AuNP-modified TRAP assay can *quantitatively* detect cancer cells over a wide dynamic range. For example, extracts of 10 to 500 MCF7 cancer cells in a background of 1000 somatic HMEC cells produced a reproducible, monotonically increasing band at 60 bp amplified product (Figure 6, top panel, lanes 1−5), the density of which was strongly correlated with the number of cancer cells in each sample (Figure 6, bottom panel). In contrast, the traditional TRAP assay produced dramatically less telomerase PCR product (smeared bands) when employed against extracts consisting of 100 to 500 cancer cells within 1000 somatic cells (Figure 6, top panel, lanes 7 and 8), and no clear

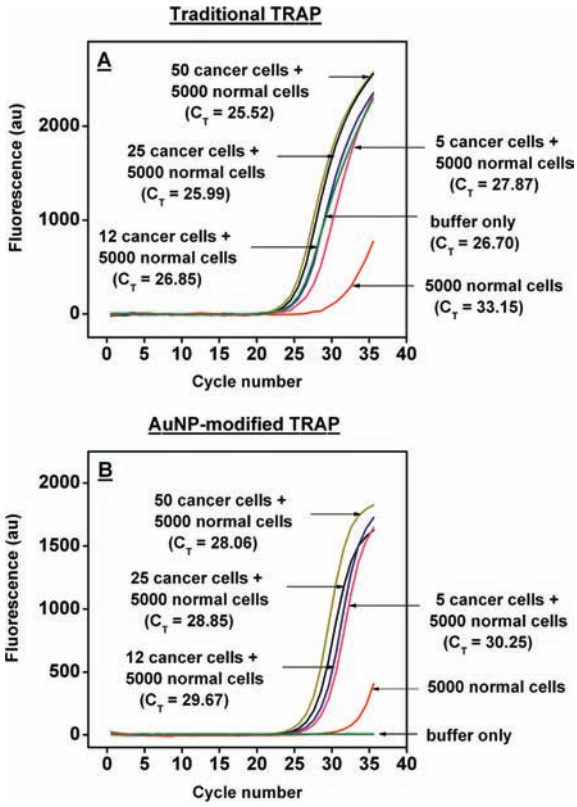

Figure 7. Real-time PCR DNA amplification curves of extracts of 50, 25, 12, and 5 cancer cells doped in extracts of 5000 somatic cells, an extract of 5000 somatic cells only, and CHAPS only using (A) the traditional TRAP assay and (B) the AuNP-modified TRAP method.

products were observed when the sample contained fewer than 100 cancer cells (Figure 6, top panel, lanes 9−11).

The detection limit of the AuNP-modified TRAP assay was further improved when employed in real-time PCR. The traditional TRAP assay performed only marginally when 50 cancer cells were doped in 5000 somatic cells ($C_T = 25.92 \pm 0.11$) and produced C_T values of 26.65 ± 0.09 and 29.20 ± 0.21 with CHAPS buffer blanks and extracts of 5000 somatic cells, respectively (Figure 7A). In contrast, the AuNP-modified TRAP assay exhibited at least 10-fold higher sensitivity than the traditional TRAP assay: it easily detected the presence of 5 cancer cells doped into 5000 somatic cells ($C_T = 30.25 \pm 0.32$) (Figure 7B) and produced no amplification upturn with either CHAPS buffer blanks or extracts of 5000 somatic cells. The modified real-time PCR TRAP assay was also highly quantitative, producing a strong correlation between the observed C_T values and the number of cancer cells in a sample (Figure S4 in the Supporting Information). In addition, we found that both TRAP assays displayed atypically high real-time PCR efficiencies in the presence of a high concentration of somatic cell extract. For example, the slopes for the standard curves of C_T versus the logarithm of the number of cancer cells were −2.78 and −2.48 for the AuNP-modified and traditional TRAP assays, respectively, which might be due to the interaction of AuNPs with proteins and PCR inhibitors from the cell extract.[29]

3.7. Generality of the TS−AuNP-Modified TRAP Assay. To ensure that the AuNP-modified TRAP assay is general and can be applied to a range of cancer cell types, we also used it to monitor telomerase activity in extracts of MDA-MB-231, MCF-7, and T-47D cells, each of which represents a distinct, immortal human breast cancer cell line, against a background of 2500 telomerase-negative somatic cells (Figure 8, lanes 1−6). As a comparison, we performed the same experiments using the traditional TRAP assay observed that under these same conditions, the traditional TRAP assay failed to detect telomerase activity in any of the three cell lines because of the large amount of primer-dimer artifacts generated in both the relevant cancer cell extracts and in CHAPS buffer and water blanks (Figure 8, lanes 7−12).

4. Conclusion

In summary, using the AuNP-modified TRAP assay as a proof of principle, we have shown that primer-modified AuNPs can significantly improve the PCR selectivity and sensitivity in complex samples. Specifically, we have shown that the AuNP-modified TRAP assay achieves exceptional sensitivity and selectivity, detecting as few as 5 cancer cells even when they are doped with up to 5000 telomerase-free somatic cells. The

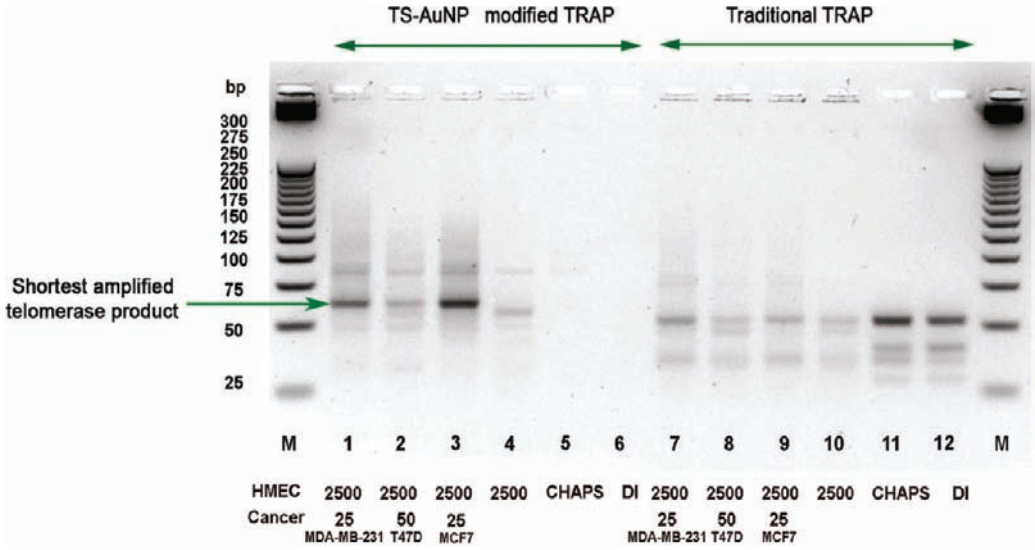

Figure 8. The AuNP-modified TRAP assay may be a fairly general approach for the detection of telomerase-active cells, enabling the detection of a number of human breast carcinoma cell lines (MDA-MB-231, T47D, and MCF7) when doped against a large excess of telomerase-negative somatic HMEC cells (lanes 1−6). In contrast, the traditional TRAP assay failed to detect telomerase activity from these same cellular extracts (lanes 7−12).

modified assay is thus vastly improved relative to the traditional TRAP assay, for which the very best reported sensitivity and selectivity is 10 cancer cells diluted only 50-fold by somatic cells.[13] The origins of the enhanced selectivity and sensitivity of the AuNP-modified TRAP assay are unclear, but the enhancements may arise from the large surface-to-volume ratio of the AuNPs,[39] which would enhance the adsorption of proteins from the cell lysate onto the particle surface.[39] Such protein adsorption indirectly, but potentially significantly, dilutes potential interferants from the cell extracts, thereby reducing the inhibition of the elongation reaction of telomerase and the subsequent PCR. The use of AuNP-modified primers in the TRAP assay reduces PCR artifacts such as primer-dimers and also reduces the inhibition often observed when TRAP is performed on crude cell lysates. We believe that the use of AuNP-modified primers may prove to be of utility for a wide range of PCR-based assays, including real-time PCR, and can be a potential solution for performing PCR directly in protein-rich solutions.

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Supporting Information Available: Experimental results for the concentration of TS−AuNPs employed in the AuNP-modified TRAP assay; melting curves of extracts of cancer cells, somatic cells, mixtures of cancer cells and somatic cells, and CHAPS using the AuNP-modified and traditional TRAP assays; and PCR efficiency of extract mixtures of different cancer cells and 5000 somatic cells using the AuNP-modified and traditional TRAP assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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