## Supporting Information For

# Enhancing-Effect of Gold Nanoparticles on DNA Strand Displacement Amplifications and its Application to an Isothermal Telomerase Assay

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### Content List

**1. Materials.**

All oligonucleotides were obtained from Integrated DNA Technologies. The deoxynucleotide solution mix, *Bst* 2.0 WarmStart DNA polymerase (8,000 units $mL^{-1}$), *Bst* polymerase large fragment (8,000 units $mL^{-1}$), nicking endonuclease Nt.BspQI (10,000 units $mL^{-1}$), deoxyribonuclease (DNase I) (RNase free, 2,000 units $mL^{-1}$), proteinase K (20 mg $mL^{-1}$), NEB3 buffer (10×), isothermal amplification buffer (10×), and quick-load low molecular weight DNA ladder were purchased from New England Biolabs Ltd.. SYBR Green I (×10,000) and SYBR Gold nucleic acid gel stain (×10,000) were obtained from Molecular Probes, Inc.. Ribonuclease (RNase) (DNase-free) (0.5 μg $\mu L^{-1}$) was purchased from Roche Applied Science. Au colloids (10 nm) were purchased from Ted Pella, Inc. It should be noted that the unit activity of the Nt.BspQI NEase may vary between different lots; the specificity of the amplifications (especially the degree of RE-pol DNA synthesis) might vary from lot to lot. Therefore an optimization should be performed before using a new lot number and, in some bad conditions, an additional 20 mM NaCl will be required for the reaction buffer.

Magnesium chloride ($MgCl_2$) solution (1 M), TWEEN 20, potassium chloride (KCl), *ethylene glycol tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), trizma hydrochloride pH 7.5 and pH 8.3 were obtained from Sigma-Aldrich.* TRAPeze® 1× CHAPS Lysis Buffer and TRAPeze® Telomerase Positive Control Cell Pellet (HeLa cancer cells) were purchased from Millipore. Cell pellets of MDA-MB-231, UM-UC-3, HCC-38, LAI-55n, and MRC-5 cell lines were kindly provided by Prof. Chuan He from The University of Chicago. 30% acrylamide and bis-acrylamide solution (19:1), ammonium persulfate (APS), tetramethylethylenediamine (TEMED), 5× nucleic acid sample loading buffer (premixed for nondenaturing TBE gels, pH 8.0) and 10× tris/boric acid/EDTA (TBE) buffer were purchased from Bio-Rad. Nanosep® centrifugal device with Omega membrane, MWCO 3kDa, was obtained from Pall Corporation. The water used in this paper was purified by BarnsteadTM Nanopure® Ultrapure Water Purification System.

**2. The Assay Protocol.**

A traditional amplification reaction (20 μL) contains variable amounts of cell extract/TPC8 (the synthetic template with eight telomeric repeats used as a positive control), the nicking telomerase substrate (NTS) primer and the nicking fluorescent reporter probe (NFRP) primer (100 nM each), Tris-HCl (30 mM, pH 8.3), $MgCl_2$ (1.5 mM), KCl (100 mM), EGTA (1 mM), Tween 20 (0.05%, vol/vol), dNTPs (200 μM each), *Bst* 2.0 WarmStart DNA polymerase (0.96 units) and Nt.BspQI NEase (5 units). Typically, the stock solution of NFRP primers (4 μM) in tris-HCl buffer (pH 7.5, 10 mM) was incubated at 90 °C for 2 min and then allowed to cool slowly to room temperature to form the hairpin structures. A master mix containing everything but cell extract/TPC8 template was assembled first on ice; Aliquoted 19 μL of the master mix into each PCR tube, added cell extract/TPC8 (1 μL) with different concentrations into corresponding tubes, and mixed well before the test. The assay was performed with incubations lasting 10-30 min at 30 °C (for telomerase extension) before initiating the DNA amplifications at 55 °C.

The assays were monitored by real-time fluorescence at an interval of 10 s. Following the amplification reaction, each 20 μL reaction was mixed with 5 μL loading buffer on ice and 10 μL of the mixture was analyzed by electrophoresis in 0.5×TBE buffer on a 12% polyacrylamide nondenaturing gel (1.5 h, 17 V $cm^{-1}$). After electrophoresis, the gel was stained in 1×SYBR Gold (in TE buffer) for 30 min, washed by pure water and visualized by the gel imager.

**3. Preparation of Cell Extracts.**

All the cells were stored as pellets at -80 °C until extraction. Each cell pellet (containing one million cells) was suspended in 200 μL CHAPS lysis buffer, incubated on ice for 30 min and then centrifuged at 12,000×g for 20 min at 4 °C. After centrifugation, 160 μL of the supernatant was transferred into a fresh tube and a series of diluted solutions in CHAPS lysis buffer were quickly prepared, flash-frozen, and stored at -80 °C before use. The complex, protein-rich samples comprise cancer cell extracts (various concentrations equivalent to 10 cells $\mu L^{-1}$ – 200 cells $\mu L^{-1}$) and a high concentration of cell lysates of foreign normal cells (MRC-5, 4,000 cells $\mu L^{-1}$ or 1,000 cells $\mu L^{-1}$), which were prepared by doping certain amounts of cancer cell extracts into normal cell extracts; the concentrations of cancer cell extracts in the complex sample and its corresponding pure sample were kept the same.

**4. Instruments.**

All the assays were performed on the CFX96 touch real-time PCR detection system (Bio-Rad) using 0.2 mL low profile PCR tubes (white). Eppendorf centrifuge model 5430R and thermomixer® R dry block heating and cooling shaker were used. The polyacrylamide gel electrophoresis (PAGE) was run on a PROTEIN II xi cell system (Bio-Rad) using the PowerPac HV as power supply (Bio-Rad) and the gel was visualized by using the Gel Doc XR+ imaging system (Bio-Rad). The UV-Vis spectra were monitored by using Synergy H4 Hybrid multiwell Plate Reader (BioTek).

**5. The traditional EXPIATR assay**

The principle of the EXPIATR assay is illustrated in Figure S1. It is a method based on a system of two primers, the NTS primer and the NFRP primer, and three enzymes, Bst 2.0 WarmStart DNA polymerase, Nt.BspQI NEase, and telomerase extracted from crude cancer cells. The NTS primer contains a non-telomeric sequence (black color) which telomerase can recognize as a substrate, and the NFRP primer, in a hairpin structure, carries a single-stranded (ss)DNA tail (brown color) which can bind to the telomeric repeat sequence. Through incorporation of the Nt.BspQI recognition sequence (red color) in both primers, the DNA amplification can be performed at a fixed temperature via the activity of the Nt.BspQI NEase to cut one strand of a double-stranded (ds)DNA at the recognition site, and the ability of the Bst 2.0 polymerase to extend the 3' end at the nick and displace the downstream strand. In the presence of telomerase, a minor number of NTS primers are converted to telomerase extension products (telomeric repeats are synthesized onto the 3' ends of NTS primers), which will act as templates for the following DNA amplification process. The hybridization between the extended NTS telomeric template and the NFRP primer initiates polymerization, resulting in the formation of double-stranded recognition sites for Nt.BspQI NEase. After the NEase cleaves the primer strands, further polymerization proceeds continually from the nick and displaces the previously copied strands. In the first stage, duplex I forms from the telomerase extension products, after which the DNA reaction is repeated in both directions, continuously releasing strands T1 and T2. In the next stage, as NTS and NFRP primers are present in excess, strands T1 and T2 can further hybridize with the free NTS and NFRP primers respectively, forming duplexes II and III which allow the DNA reactions to occur in a single direction. The cycling reaction between II and III finally results in the exponential amplification of telomerase products, in which strands displaced from duplex II (same as T1) serve as targets for the NTS primer (to form the new duplex III), while strands displaced from duplex III (same as T2) serve as targets for the NFRP primer (to form the new duplex II). As the NFRP primer can bind multiple positions along the templates, according to the number of the telomeric repeats extended on the NTS primers, the amplification reaction yields a series of lengths of duplex II and duplex III (which have the identical lengths). At the final stage, when the NTS and NFRP primers become limiting, the further nicked duplex IV (T1•T2) starts to accumulate in the reaction, which will be 18 bps shorter than duplex II and duplex III.

The sequences of the oligomers are as follows:

NFRP (/5IABkFQ/AGC AGG AAG CGC TCT TCC TGC /iFluorT/CC CTA ACC CTA ACC C) /5IABkFQ/ = 5' Iowa Black® FQ; /iFluorT/ = Int Fluorescein dT.

NTS (GTG CGT GAG AGC TCT TCC AAT CCG TCG AGC AGA GTT)

TPC8 (GTG CGT GAG AGC TCT TCC AAT CCG TCG AGC AGA GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG G)

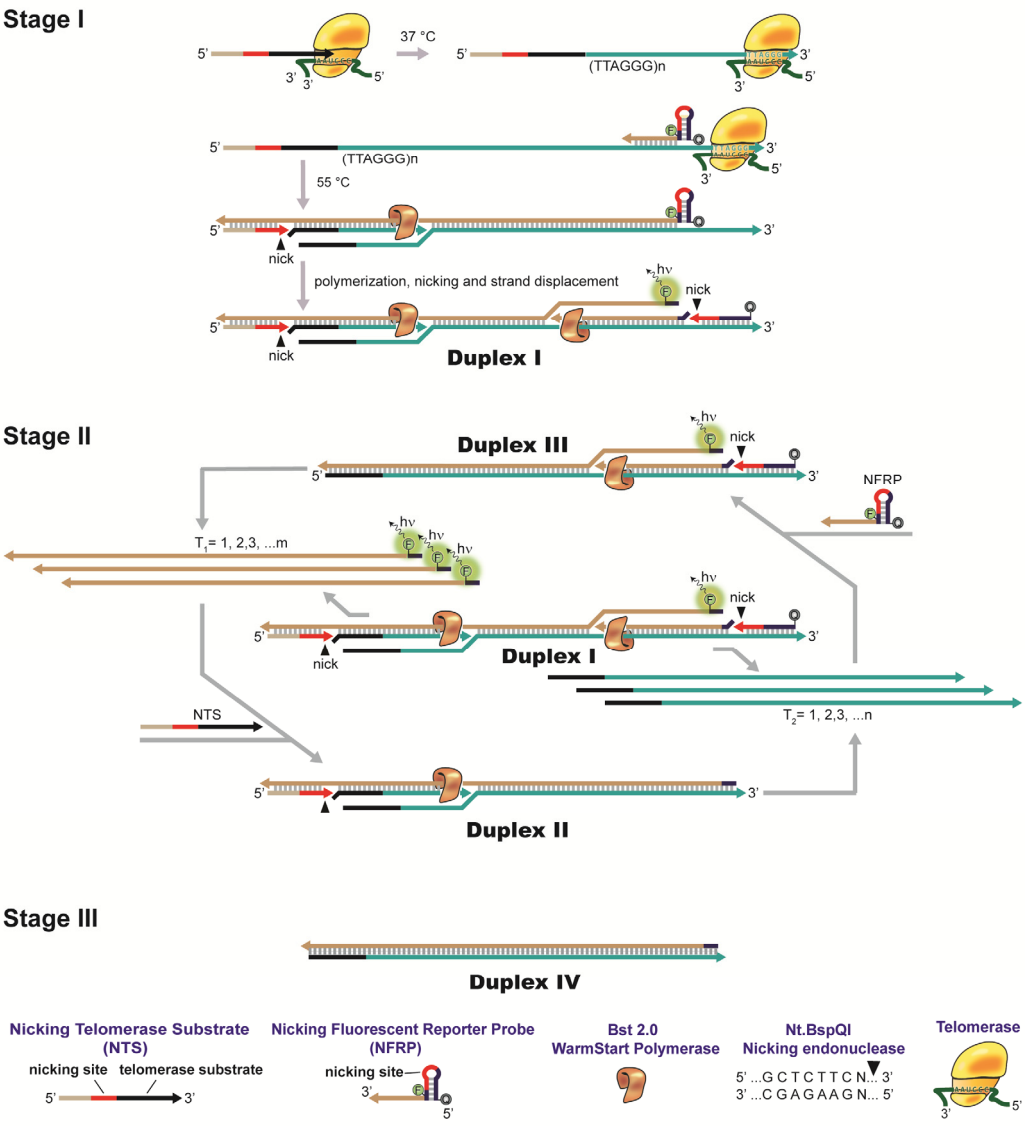


**Figure S1. A schematic diagram of the EXPIATR assay.**

**6. Different cell lines were tested by the EXPIATR assay and their relative telomerase activities were quantified.**

The telomerase activity of a given sample was quantified based on a relative standard curve method referenced from the TRAP assay.[1] Generally speaking, telomerase activity is expressed relative to a standard sample by this method. In our assay, the HeLa cancer cell (which shows medium to high telomerase activity) was chosen as a standard. The telomerase activity of an unknown sample was evaluated by comparison with the standard curve of the HeLa cell line and expressed by the number of HeLa cancer cells that can generate the same level of signal as the unknown sample. In addition, each test was normalized by the positive control ($10^{-11}$ M TPC8) performed in the same experiment. The equation is:

$RTA = 10^{a}$

$$a = \left( POI_{sample} \cdot \frac{POI_{IR\ standard}}{POI_{IR\ sample}} - Y_{int} \right) / slope$$

RTA: the relative telomerase activity;

$Y_{int}$: the Y-intercept of the standard curve of the standard assay based on HeLa cancer cell;

Slope: the slope of the standard curve of the standard assay based on HeLa cancer cell;

$POI_{sample}$: the POI value of the amplification curve of an unknown sample;

$POI_{IR\ standard}$: the POI value of the amplification curve of TPC8 positive control in the standard assay;

$POI_{IR\ sample}$: the POI value of the amplification curve of TPC8 positive control in the assay of unknown sample.

Different cell lines, cervical cancer (HeLa), breast cancer (MDA-MB-231 and HCC-38), bladder cancer (UM-UC-3), neuroblastoma (LAI-55n), and lung fibroblast normal cells (MRC-5) were tested and their telomerase activities were quantified. MRC-5 was found telomerase-negative; HeLa, MDA-MB-231, HCC-38 and UM-UC-3 displayed moderate to high telomerase activities, which is in accordance with the results of other telomerase detection methods.[2] The neuroblastoma cell line LAI-55n showed a relatively low RTA, i.e. the extract from 200 LAI-55n cells exhibited telomerase activity equivalent to the extract from ~ 8 HeLa cancer cells (Figure S2). Although LAI-55n showed much lower telomerase activity than HeLa, the standard curves for both HeLa and LAI-55n are linear over the tested cell number range (from 200 to 1 cell), suggesting that EXPIATR is a specific and sensitive method for telomerase detection.

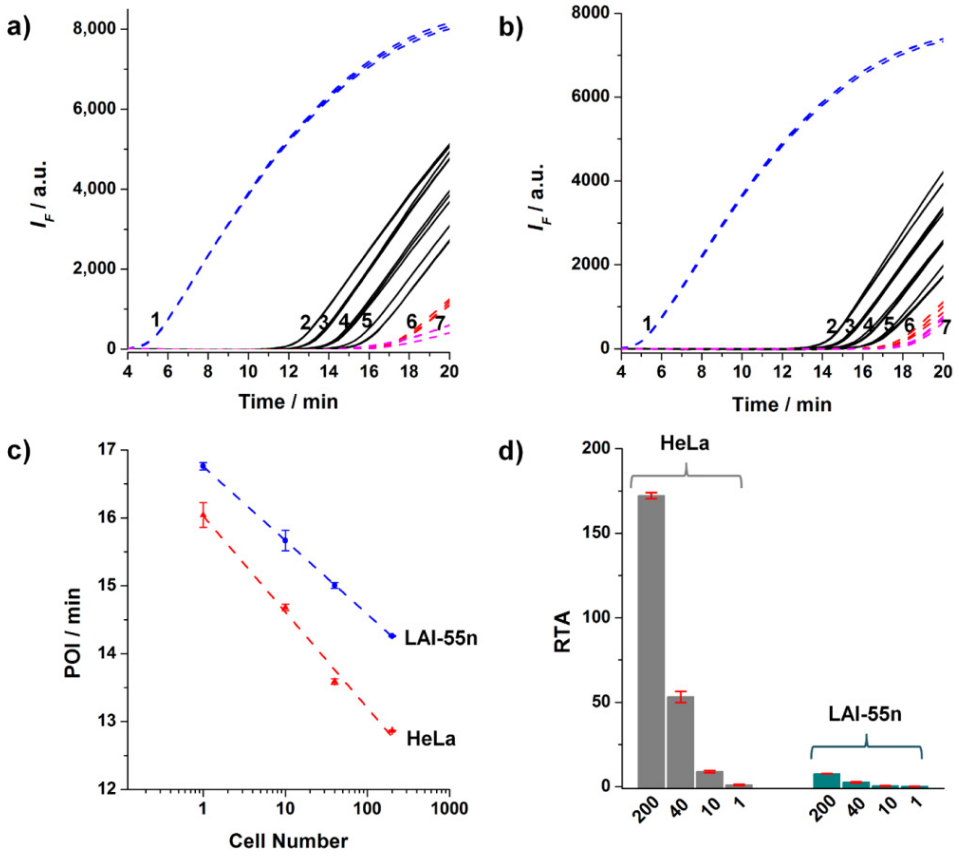


**Figure S2.** Detection of telomerase activities in human HeLa cancer cells and in human LAI-55n neuroblastoma cells by the EXPIATR assay. (a) The assay tested on HeLa cell extracts and (b) the assay tested on LAI-55n cell extracts included: the amplification curves of TPC8 positive control ($1\times10^{-11}$ M) (1), 200 cells, 40 cells, 10 cells and 1 cell equivalent extracts (2-5), negative control in absence of cell extracts (6), and heat-inactivated extracts (1000 cells equivalence) (7); (c) Standard curves of the assay for HeLa cell extracts (1) and the assay for LAI-55n cell extracts; (d) The RTAs of HeLa cells and LAI-55n cells. Error bars indicate standard error of triplicate tests.

## 7. Optimizing the concentration of AuNPs in the EXPIATR-AuNP assay

The optimal concentration of 10 nm citrate-capped AuNPs for the EXPIATR-AuNP assay was assessed by the amplifications of TPC8 template ($1\times10^{-13}$ M). As shown in Figure S3, the solid curves correspond to amplifications of TPC8 template in the presence (red) and absence of (black) concentrated cell lysates equivalent to 4000 normal cells; while the dashed curves correspond to non-template controls in the presence (red) and absence of (black) normal cells. From top to bottom, different concentrations of citrate-capped AuNPs (from 0 to 0.5 nM) were mixed with the reactions to study the concentration effect of AuNPs. In the absence of AuNPs, the amplification curve of TPC8 template showed a delayed POI value when cell lysates were presented in the reaction, implying the amplification reaction was inhibited by the cell lysates. Along with the addition of more AuNPs, the inhibitory effect of cell lysates on the amplification reactions was gradually reduced. Meanwhile, the amplifications of pure TPC8 template would not be significantly affected until the concentration of AuNPs reached 0.4 nM; however, the amplification rates (efficiency) started to decrease significantly when more than 0.4 nM AuNPs were used. It is in accordance to previous reports about PCR that an appropriate concentration of nano-materials may improve PCR, but an excessive concentration may inhibit the amplification process.[3] To make the method compatible to the tests of complex samples but also pure samples. From Figure S3, it demonstrates that 0.4 nM AuNPs should be the optimal concentration for the EXPIATR-AuNP assay.

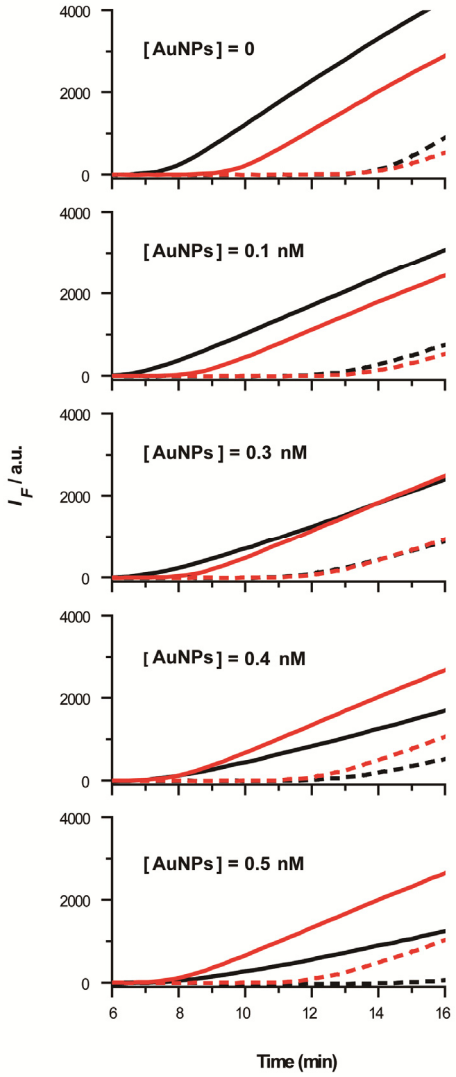


**Figure S3.** The concentration effect of 10 nm citrate-capped AuNPs on the assay was assessed by the amplification reaction of TPC8 template ($1\times10^{-13}$ M) (solid) and by the corresponding non-template controls (dashed), in the presence (red) and in the absence of (black) concentrated cell lysates equivalent to 4000 normal cells.

**8. The gold colloid was purified to prove that the AuNPs are the effective components.**

Due to the purchased citrate-capped AuNPs were suspended in a cocktail solution containing some impurities besides gold nanoparticles,[4] it is necessary to further verify that the AuNPs are the essential component working for the EXPIATR-AuNP assay. We used the Nanosep centrigugal device with Omega membrane MWCO 3 kDa to replace the storing liquid of the gold colloid solution with pure water. The purchased gold colloid (Figure S4-a), the purified gold colloid in water (Figure S4-b), and the filtrate (storing liquid of the gold colloid) collected from the Nanosep device (Figure S4-c) were used as additives to the EXPIATR assay and tested respectively. In Figure S4, the solid cyan curves are the amplifications of cell extracts of 200 HeLa cancer cells; the dashed curves are the amplifications of cell extracts of 200 HeLa cell cells doped in 4000 MRC-5 normal cells with the presence of the corresponding additives. We observed that the gold colloids both purchased and purified could suppress the inhibitory effect caused by the concentrated cell lysates on the amplification reactions, however, the filtrate collected from the centrifugal device (storing liquid of the gold colloid) could not decrease the inhibitory effect at all. It indicates that AuNPs are indeed the essential component working for the EXPIATR-AuNP assay.

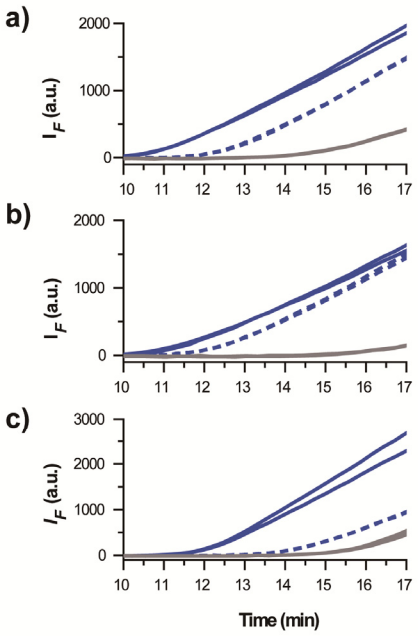


**Figure S4.** The amplifications of extracts of 200 HeLa cancer cells, in the absence of (solid cyan) and in the presence of (dashed cyan) concentrated cell lysates, as well as the corresponding non-template controls (solid grey); For the detections on complex cell extracts (dashed cyan), different additive were present: a) the purchased gold colloid, b) the purified gold colloid, and c) the storing liquid of the purchased gold colloid collected from the centrifuge device.

## 9. AuNPs working on nucleic acid amplification step rather than on the telomerization step

EXPIATR is a one-pot assay that its two reaction stages, the telomerase extension at 30 °C and the nucleic acid amplifications at 55 °C, can run sequentially in a closed-tube and not affect each other (the one-step protocol). As both stages would affect the final results of the assay, it is necessary to further study which stage of the assay is actually affected by the cell lysates and AuNPs. For this purpose, independent telomerization step was performed by incubating the cancer cell extracts with dNTPs and NTS primers at 30 °C, and then heat-inactivating telomerase from the cell extracts at 85 °C; subsequently the telomerase extension products were used as targets, directly or doped in a high concentration of foreign cell lysates (from 4000 normal cells), to run the amplification reactions (the two-step protocol). Figure S5-b shows by the two-step protocol the detections on extracts of 200 HeLa cancer cells, pure and in the presence of concentrated cell lysates (equivalent to 4000 normal cells); although not associated with the telomerization process, the cell lysates still induced negative effects to the amplification process, which could also be improved by the addition of AuNPs. All were similar to the results of using the one-step protocol (Figure S5-a). It proved that the inhibitory effect of cell lysates and the enhancing effect of AuNPs were more related to the enzymatic nucleic acid amplification process rather than the telomerization process.

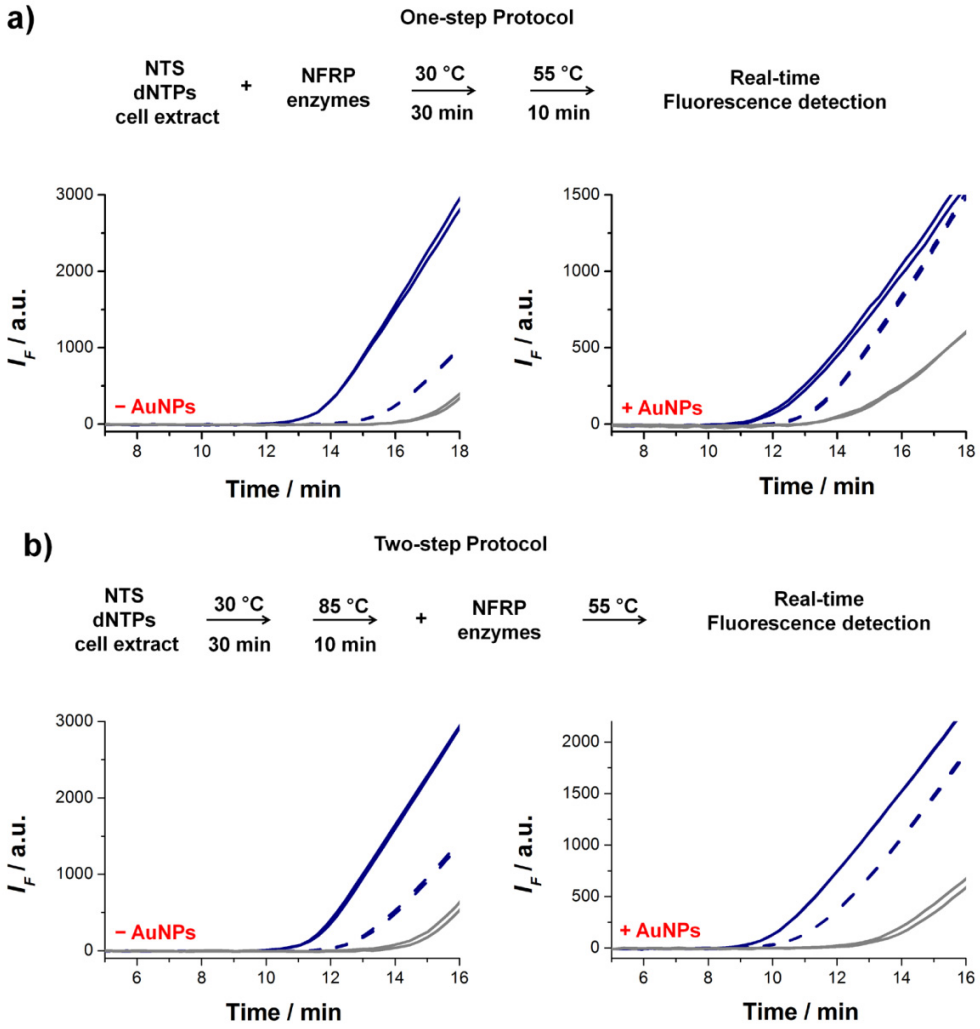


**Figure S5.** The EXPIATR assay and the EXPIATR-AuNP assay tested on extracts of HeLa cancer cells (equivalent to 200 cells), pure (solid navy) and in presence of a high concentration of cell lysates (equivalent to 4000 normal cells) (dashed navy), were performed by the traditional one-step protocol (a) or modified two-step protocol (b) respectively; the corresponding non-template controls (grey) were also presented.

## 10. The non-specific reactions of EXPIATR were studied to reveal which component of the cell lysates was the essential inhibitor to the specific amplification reactions

Tan et al. carefully studied the possible nonspecific reactions for the nicking-enzyme-mediated SDA (NE-SDA) reactions.[5] They found that the non-specific reactions included an early phase related to primer-dimer like artifacts and a late phase in general agreement with the literature reports of RE-pol (restriction-endonuclease-polymerase) DNA synthesis. The RE-pol DNA synthesis is a kind of DNA-independent synthesis, which would produce new DNA strands from dNTPs only by enzymes (both the thermophilic polymerase and the NEase) in the absence of any templating or priming DNA strands. The Primer/template-independent RE-pol DNA synthesis has been observed in many isothermal amplifications working upon varies of polymerases and NEases. To further understand the RE-pol DNA synthesis, we did several control experiments. As shown in Figure S6-a, the following conclusions were obtained: (1) In the absence of dNTPs, the *Bst* polymerase and the Nt.BspQI NEase with (lane 2) or without (lane 3) heating at 55 °C could not induce RE-pol DNA synthesis. The results are in accordance with the previous reports for RE-pol DNA synthesis in which the non-specific DNA sequences were synthesized from dNTPs by the enzymes. (2) Also in the absence of Nt.BspQI NEase (lane 4) or *Bst* polymeras (lane 5), no RE-pol DNA synthesis took place. (3) Furthermore, we checked that if the cell lysates could induce some additional RE-pol DNA synthesis. For itself (lane 6), combined with Bst polymeras (lane 7) or Nt.BspQI NEase (lane 8), cell lysates from 4000 normal cells could not synthesize DNA sequences from dNTPs, indicating the cell lysates could not induce more RE-pol DNA synthesis in the reaction system.

As a further study, we treated the cell lysates in different ways to reveal which component in the cell lysates would cause the RE-pol DNA synthesis. The non-template reactions only in presence of primers but no template were run in the un-optimal Isothermal Amplification (IA) buffer to emphasize the non-specific signals. In the presence of un-treated cell extracts equivalent to 4000 normal MRC-5 cells, both non-specific reactions of the primer-dimer like artifacts and the RE-pol DNA synthesis were observed on the gel (lane 2). Firstly, we pre-incubated the cell extracts with DNase I at 37 °C for 30 min, inactivated the DNase I at 75 °C for 10 min, and then added the treated cell extracts to the non-template amplification reaction (lane 6), at which condition the RE-pol DNA synthesis was still observed as part of the non-specific reactions. The same result was observed when the cell extracts were pre-incubated with RNase (lane 7). Further, the cell extracts were pretreated with proteinase K to degrade proteins by cleaving peptide bonds and catalyzing peptide amide hydrolysis, and then the proteinase K was heat-inactivated at 95 °C prior to mixing the treated cell extracts with the non-template reaction. Interestingly, under this condition (lane 3), the RE-pol DNA synthesis was eliminated from the non-specific reactions, and only the primer-artifacts non-specific reactions left. Lane 4 shows a control experiment in which the cell extracts underwent the same incubation process as lane 3 but without the addition of proteinase K. In this control condition without the addition of proteinase K, the RE-pol DNA synthesis occurred again, verifying the

function of proteinase K but not the incubation process was essential to depress the RE-pol DNA synthesis. Due to the truth that proteinase K cannot be completely inactivated even at 95 °C, proteinase K might cause the inactivation of Bst polymerase and as a result depress the RE-pol DNA synthesis. Therefore, in another control experiment (lane 5), a new portion of untreated cell lysates was added to the reaction of lane 3. Under this condition, the RE-pol DNA synthesis occurred as part of the non-specific reactions, indicating that the *Bst* polymerase was not badly affected (at least not totally inactivated) by the presence of proteinase K.

As a result, we found that the elimination of cellular proteins from the cell lysates recovered the specificity of the amplification system. It demonstrated that the cellular proteins were essential for inhibiting the specific amplification and facilitating the RE-pol DNA synthesis. Waleed et al. reported that proteins, like on the basis of N-terminal amino acid sequences, were prone to bind with ssDNAs and acted as inhibitors to PCR by making the DNA templates unavailable for polymerase.[6] Combined with our observations that the inhibition effect caused by cell lysates was more significant for the amplifications of low-aboundant templates, we assumed that the cellular proteins might bind with ssDNAs and make the templates unavailable for polymerase. As a result, like a competition, the specific reaction was attenuated and the DNA-independent RE-pol DNA synthesis would be boosted at the same time.

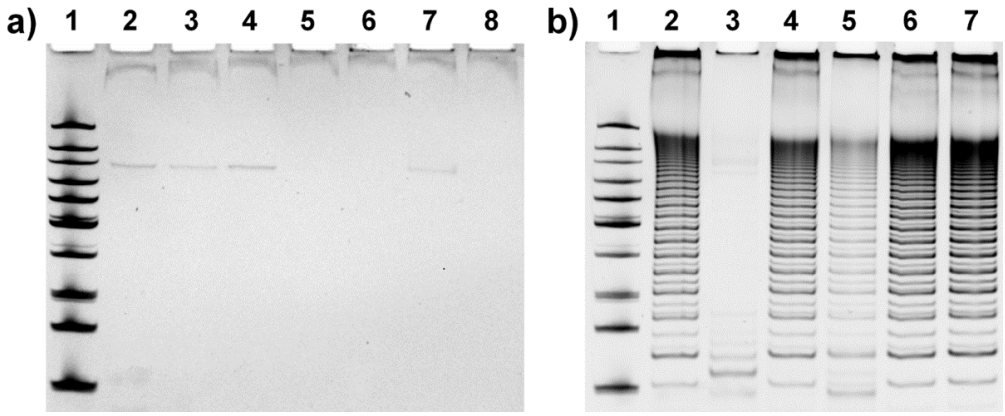


**Figure S6.** (a) The survey of the essentials for producing a RE-pol DNA synthesis: DNA ladder (lane 1), Bst polymerase + Nt.BspQI without heating (lane 2), Bst polymerase + Nt.BspQI (lane 3), Bst polymerase + dNTPs (lane 4), Nt.BspQI + dNTPs (lane 5), cell lysates + dNTPs (lane 6), cell lysates + Bst polymerase + dNTPs (lane 7), and cell lysates + Nt.BspQI + dNTPs (lane 8); the reactions in Lane 3 – Lane 8 were heated at 55 °C for 15 min prior to the gel characterizations; (b) The cell lysates with some pretreatments and their influences to the non-template reactions (in the IA buffer): DNA ladder (Lane 1), the non-template reactions in presence of: cell lysates without any treatment (lane 2), cell lysates with proteinase K treatment (lane 3) and its two control experiments (Lane 4 and Lane 5), cell lysates with DNase treatment (lane 6), and cell lysates with RNase treatment (lane 7).

## 11. The negligible quenching effect of AuNPs on the fluorescence of the NFRP probe

As shown in Figure S7, the thermal denaturation profile of the NFRP-primer fluorescence probe was measured by increasing the temperature from 40 °C to 95 °C in increments of 2 °C (each temperature point was held for 2 min). In the presence of 0.4 nM AuNPs, only negligible quenching effect was observed.

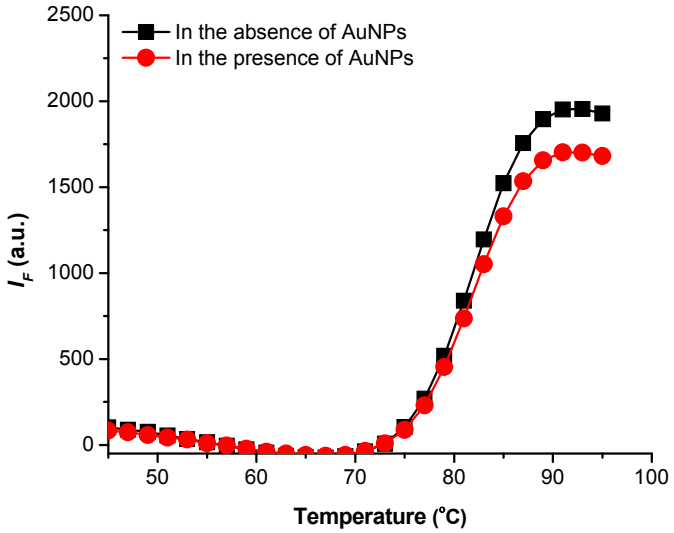


**Figure S7.** Thermal denaturation profile of NFRP-primer fluorescence probe in the absence of AuNPs (black) and in the presence of AuNPs (red).

**12. The concentration of AuNPs has to be optimized for the EXPIATR-AuNP assay.**

The UV-Vis spectroscopic study (Figure 7 in the maintext) revealed that the enzymes showed a stronger affinity to AuNPs compared with the cellular proteins of the cell lysates, however the presence of a high concentration of cellular proteins would still affect the effectiveness of AuNPs in enhancing the amplification reactions as they would occupy parts of the nanoparticle surfaces. Therefore we observed that when cell lysates were abundant in the reaction mixtures, AuNPs only marginally suppressed the primer-artifact amplification and showed noticeably less improvements in the specific amplifications. As shown in Figure S8, the non-template control in the absence of cell lysates was significantly inhibited when more than 0.4 nM AuNPs was added to the amplification system, and the assay performed on the normal cell lysates (equivalent to 4000 MRC-5 cells), with a high abundance of cellular proteins, exhibited a higher amplification rate than the non-template control, resulting in the false-positive problem. The problem with the use of additional AuNPs is that the amplification in the presence of cell lysates would be benefited, because more AuNPs would conquer the influence from cell lysates and maximize the effectiveness of the AuNPs to the amplification reactions and meanwhile the cellular proteins would bind parts of the nanoparticle surfaces and protect the enzymes from excessive binding; on the contrary, the amplification in the absence of cell lysates would be inhibited by the same amount of AuNPs as the excessive binding of the enzymes to AuNPs would contrarily decrease their activities.[3] Thus considering removing the false-negative problem induced by cell lysates as much as possible and not inducing new false-positive problem, 0.4 nM AuNPs, a concentration showing negligible effect to the amplification in the absence of cell lysates, is optimized for the modified EXPIATR-AuNP assay.

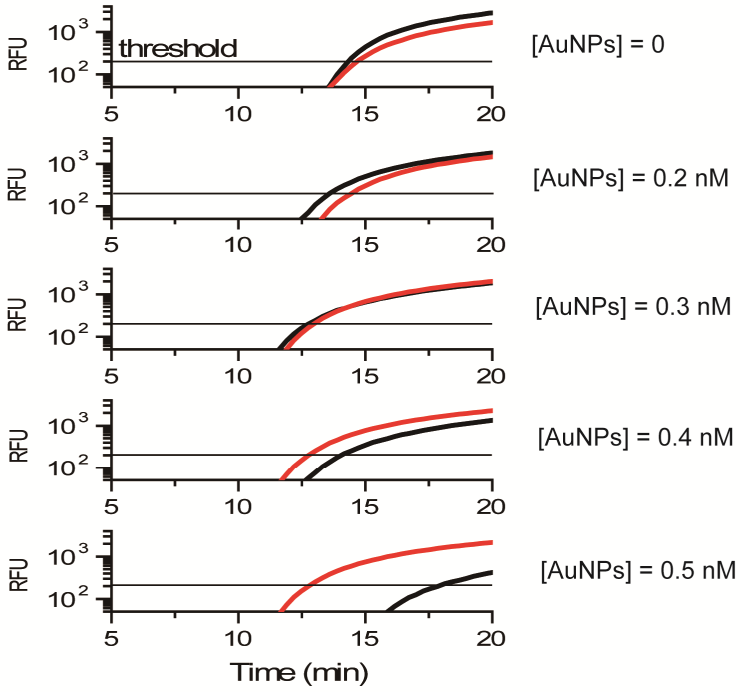


**Figure S8.** The concentration effect of 10 nm citrate-capped AuNPs for the EXPIATR-AuNP assay: the non-template control in the absence of cell lysates (black) and the detection on normal cell lysates (equivalent to 4000 MRC-5 normal cells) (red).

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