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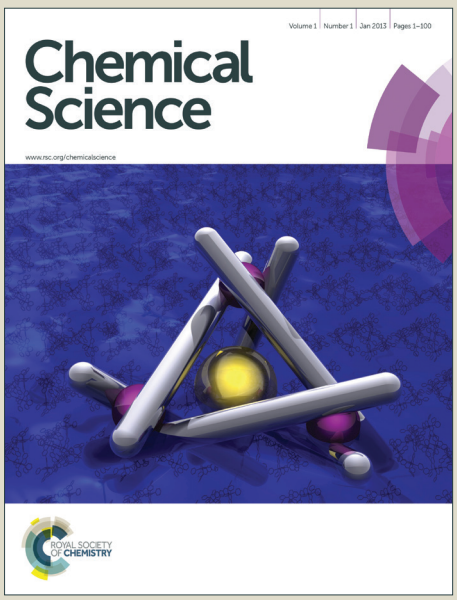

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ARTICLE

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

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The exciting applications of gold nanoparticles (AuNPs) in bio-diagnostics are not limited to use as signal transduction probes; the fascinating effect of AuNPs on enhancing the reactions of nucleic acids has also been increasingly recognized. Herein, we explored the power of AuNPs and their enhancing effect on isothermal amplification reactions. The EXPIATR assay, based upon a programmed path of isothermal strand-displacement-amplifications, has been proven to be a sensitive assay for telomerase activity. However, the assay is not applicable to the complex, protein-rich samples which more closely resemble real clinical specimens, since the abundant cellular proteins in the complex samples can impair the specificity of the amplification reactions. In the presence of AuNPs, the sensitivity of the detection of telomerase activity in complex samples is improved five-fold compared with the traditional assay, providing an efficient way to enhance the reliability of the EXPIATR assay to a new level. In addition to the situation of low-specificity caused by the external interference of cell lysates, it was further demonstrated that AuNPs showed a similar effect on improving the low-specificity caused by polymerase, which implies that AuNPs affect the amplification reactions in a very fundamental way, presumably by enhancing the activity and stability of the amplification enzymes. By revealing and demonstrating the beneficial role of AuNPs in nucleic-acid reactions, this study provides a new avenue to promote the clinical applications of isothermal nucleic acid amplification.

Introduction

Nucleic acid amplification (NAA) techniques are basic tools in molecular biology with widespread applications in biology and medical research.[1] Featuring unprecedented sensitivity, these amplification techniques generally use repeated enzymatic reactions to make numerous copies of a sequence initially present at a low concentration. The most widely used NAA technique is PCR, which is powerful but not without limitations.[2,3] Some isothermal NAA methods with higher flexibility for bio-diagnostics using strategies distinct from PCR have been developed.[4] These methods can reach specificity and sensitivity equivalent to PCR and have no need for the thermal cycling protocol. Thus, they simplify some special requirements allowing for a wider range of applications, such as point-of-care diagnosis,[5] *in situ* detection,[6] on-chip detection,[7] and naked-eye detection.[8] Also, some of the isothermal methods are more adaptable to specific biomolecular targets for which PCR would have to use very sophisticated protocols. For example, short-length microRNA sequences could be directly amplified and detected by the isothermal exponential amplification reaction (EXPAR)[9] method, skipping the reverse transcription step required by PCR. The isothermal NAA methods show attractive potentials and act as the perfect complement to the PCR technique; however, they suffer the problems common in enzymatic reactions,[10] being sensitive to contaminations and intolerance to the inhibitory components from crude samples, limiting their reliability in clinical applications. In particular, the knowledge of these isothermal NAA methods, especially in regards to specificity, is still not as comprehensive as that of PCR, which further impedes their applications in clinical practice.

The isothermal nicking enzyme-mediated strand displacement amplification (NE-SDA) uses a restriction enzyme to nick at a specific site and a polymerase to initiate a new cycle of replication from the 3′-OH end of the nick, which can exponentially amplify the target sequence at a fixed temperature.[11-15] Recently, a real-time *in vitro* detection method of telomerase activity, exponential isothermal amplification of telomere repeat (EXPIATR),[16] was developed based upon a programmed path of NE-SDA reactions. Although EXPIATR

and some other NAA methods for telomerase detection,[17-23] like the PCR-based telomeric repeat amplification protocol (TRAP), can reach the excellent sensitivity of a single cancer cell, their reliability in clinical diagnosis are still questioned. One potential problem is the risk of generating false-negative results due to the presence of inhibitors of the amplification reactions in the analysis of total protein cell extracts.[24] As telomerase activity appears early in pathogenesis of many cancers, telomerase is especially important for the early detection of cancer.[25] The newly developed methods prefer using some minimally-invasive or non-invasive samples, like biopsy specimens and body-fluid samples for early detections,[26,27] which could require the assay to be applied to samples with a minute amount of cancer cells in a background of hundreds or even thousands of normal cells. This reveals more challenges for telomerase detection; besides the high sensitivity, a good specificity is required to minimize the influences from the background. The TRAP assay, which has been evaluated in clinical studies, attempted to overcome the false-negative problem by removing the inhibitors from samples prior to the amplification process *via* phenol/chloroform extraction,[28] biotin affinity labeling and extraction,[29] or by using an "internal standard" DNA strand as a reference to monitor the efficiency of the specific amplification.[30] However, these methods complicate the simplicity of the assay and increase the risk of introducing carry-over contaminations.

Some pioneering work has been done using AuNPs to improve the PCR performance in different error-prone systems.[31-36] For example, Fan and coworkers found that AuNPs could enhance the specificity of the allele-specific PCR for single nucleotide polymorphism (SNP) genotyping and haplotyping.[36] Xiao *et al*. used primer-modified AuNPs to perform the TRAP assay and observed that such a modification could significantly improve the selectivity and sensitivity of telomerase activity detection on complex samples.[19] Here we report the effect of AuNPs on improving the specificity of isothermal NE-SDA reactions and resolving the issues of the EXPIATR assay when performed on complex, protein-rich samples. In addition, this paper provided more evidences to validate the role of AuNPs in enhancing the reactions of nucleic acids, as according to our knowledge, the AuNP effect has not previously been studied in an isothermal amplification system. First the isothermal NE-SDA reactions under different situations of low-specificity, either caused by the external interference of cell lysates or caused by polymerase, were investigated through the addition of AuNPs. The power of AuNPs in enhancing and reforming the NE-SDA reactions has been well demonstrated; furthermore, it was revealed that the addition of AuNPs could also inhibit the non-specific reactions, especially for the restriction-endonuclease-DNA-polymerase (RE-pol) DNA synthesis. As a ubiquitous non-specific reaction for NE-SDAs, the RE-pol DNA synthesis is the DNA-independent synthesis which produces new DNA strands from dNTPs using only enzymes but not requiring any templating or priming DNA strands. This kind of non-specific reaction has not been well understood and lacks efficient suppressing methods, resulting in a major impediment to the practical applications of the NE-SDA methods.[13] A recent report found that the single-stranded DNA binding protein T4 bacteriophage gene 32 (T4gp32) could inhibit the RE-pol DNA synthesis;[37] our observation proved that AuNPs might also be inhibitors to this poorly-understood non-specific reaction. Also, thanks to the sensitive response of the surface plasmon of AuNPs to the environment, the interaction between AuNPs and the reaction components was monitored by UV-Vis spectroscopy, which aided in further understanding the way AuNPs influence the amplification reactions and in tuning the concentration of AuNPs. Taking advantage of such AuNP effects, the EXPIATR assay could overcome the inhibitory effect induced by concentrated cellular proteins and, as a result, maintain its sensitivity on complex, protein-rich samples; for example, for complex samples of various cancer cells in the presence of 100-fold foreign normal cells, the detection sensitivity achieved by the AuNP-based assay was significantly improved, an increase by as much as five-fold, as compared with the traditional assay. Moreover, besides making the EXPIATR assay applicable for complex clinical samples, this study shows broad significance in understanding the essential role of AuNPs in enhancing the isothermal nucleic acid amplifications and promoting their clinical applications.

Results and discussion

The EXPIATR[16] assay recently developed by our group is a sensitive assay for telomerase, and has been verified by tests on pure cancer cells from established cell lines (Supporting Information, Fig. S1 and Fig. S2). However, more challenges will be faced before advancing to further clinical practice, as clinical samples are more complex. The TRAP assay has met the problem of the complex matrix of clinical samples which contain inhibitors of the amplification reactions and yield false-negative results.[24] To further characterize the capability of the EXPIATR assay, it was tested on complex samples which were enriched with cell lysates of normal cells. For the complex samples containing a certain amount of cancer cell extracts (from HeLa cell line) and the extracts of 1000 foreign cells (from MRC-5 cell line), the real-time amplification curves showed obvious differences from that of the corresponding pure samples containing the same amount of cancer cell extracts (Fig. 1a). The transformation of the real-time results into the quantitative relative-telomerase-activity (RTA) values revealed that only 20% of the telomerase activity of the pure HeLa cancer cell extracts was detectable for the complex samples containing 1,000 normal cells (Fig. 1c). When the interferants in the complex samples were further increased to a total protein extract from 4,000 foreign cells, the detections of telomerase activity in such complex, protein-rich samples were significantly inhibited (Fig. 1b); less than 5% of the telomerase activity of cancer cells could be expressed in the complex samples containing 4,000 normal cells. All these results indicate that the sensitivity of the EXPIATR assay was greatly affected by the presence of foreign cell lysates in a given sample; the more foreign cell lysates included, the more

inhibited the assay would be, in comparison with the assay performed on pure cancer cells. A similar effect was observed with the TRAP assay in which the addition of 5,000 normal cells would almost completely inhibit the amplification of the elongated telomerase products.[19]

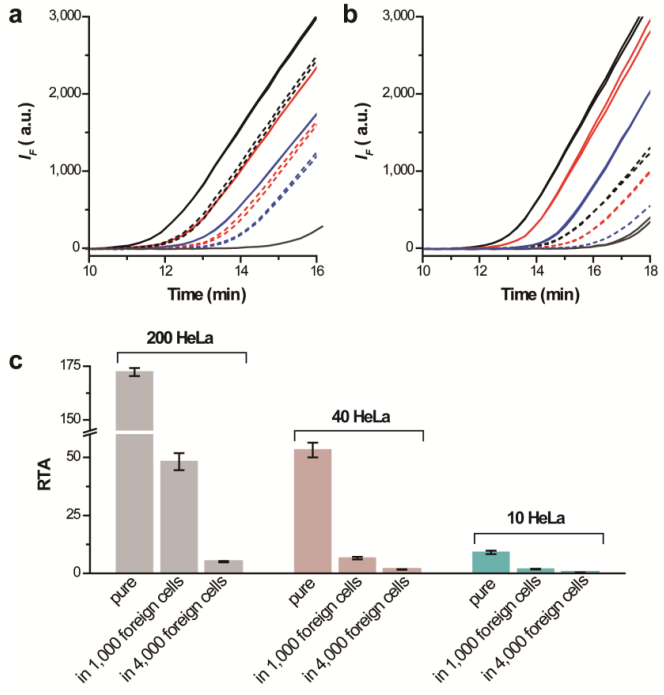

Fig. 1 Real-time EXPIATR assay performed on the extracts of HeLa cancer cells, pure (solid) and in the presence of a high concentration of foreign normal cell lysates (dashed): 200 cancer cells (black), 40 cancer cells (red), 10 cancer cells (blue), in the presence of extracts of 1,000 normal cells (a) and in the presence of extracts of 4,000 normal cells (b), and the non-template controls were also present (solid grey) (duplicate measurements of each concentration); The real-time results are transformed to quantitative RTA values and summarized (c). Error bars indicate standard error of triplicate tests.

As telomerase detection is carried out on cellular protein extracts, any purification strategy to diminish the inhibitory effect of interfering cell lysates has to be performed after the telomerization step. A pre-amplification step would increase the operation complexity and reduce the reliability of the results. Alternatively, several studies have revealed that gold colloids improve the performance of PCR in a wide variety of error-prone systems;[31,32,36] more importantly, this AuNP-based method is ready-for-use with no need for making significant changes to the original protocols. Hence, we developed an AuNP-based assay with the addition of 0.4 nM citrate-capped AuNPs to the traditional EXPIATR assay (Supporting Information Fig. S3). Fig. 2 shows the real-time results of employing the AuNP-based assay to detect HeLa cancer cells alone and in presence of foreign matters. In the presence of extracts of 1,000 foreign normal cells, the complex samples produced almost identical amplification signals to the corresponding pure samples (Fig. 2a), indicating the inhibitory effect from 1,000 foreign cells was almost completely eliminated by the addition of AuNPs. Even for the complex samples with the presence of 4,000 foreign cells, the performance of the AuNP-based assay was significantly improved compared with that of the traditional assay (Fig. 2b), *i.e.* >20% of the telomerase activity of cancer cells can be expressed in the complex samples (Fig. 2c), in contrast to < 5% detectable by the traditional assay. As a further test, we checked different cancer cell lines in the presence of a 100-fold excess of foreign cell extracts (*i.e.* extracts from 40 cancer cells were doped in the extracts of 4,000 normal cells) by the AuNP-based assays (Fig. 3). For all the cancer cell lines, the addition of AuNPs significantly improved the sensitivity for telomerase detection in complex samples, over five-fold as compared with the traditional assays. As the citrate-capped AuNPs were suspended in a cocktail solution which may contain some impurities besides gold nanoparticles, further experiments confirmed that the functional parts of the purchased AuNP colloid were the nanoparticles themselves, not the impurities in the solution (Supporting Information Fig. S4).

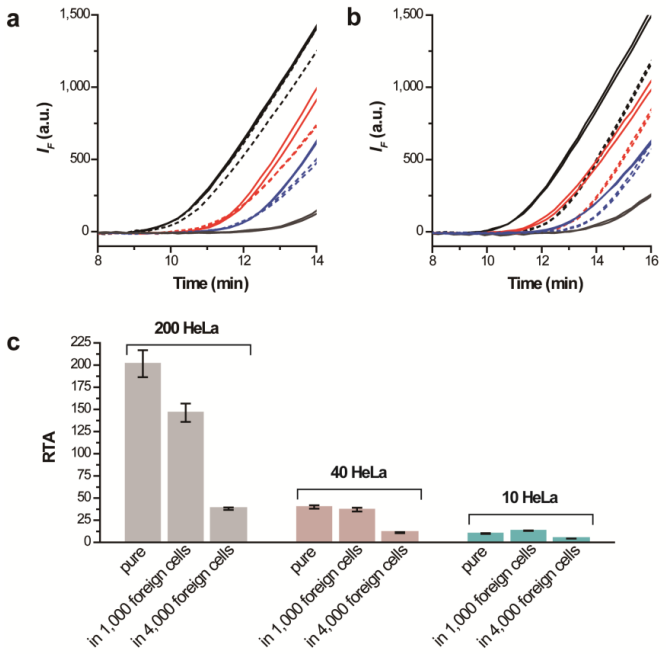

Fig. 2 Real-time EXPIATR-AuNP assay performed on the extracts of HeLa cancer cells, pure (solid) and in the presence of a high concentration of foreign cell lysates (dashed): 200 cancer cells (black), 40 cancer cells (red), 10 cancer cells (blue), in the presence of extracts of 1,000 normal cells (a) and in the presence of extracts of 4,000 normal cells (b), and the non-template controls were also present (grey solid) (duplicate measurements of each concentration); The real-time results are transformed to quantitative RTA values and summarized (c). Error bars indicate standard error of triplicate tests.

To further study of the roles of cell lysates and AuNPs, the amplified products collected from different assays were visualized by polyacrylamide gel electrophoresis (PAGE) (Fig. 4). The assays were monitored by real-time fluorescence, quenched shortly after the non-template controls started to generate signals, and applied to the PAGE analysis directly. According to the principle of EXPIATR (Supporting Information, Fig. S1), a ladder of products with 6 bp increments will be produced. Fig. 4a shows the assays tested on complex samples containing 1,000 normal cell lysates and Fig. 4b shows

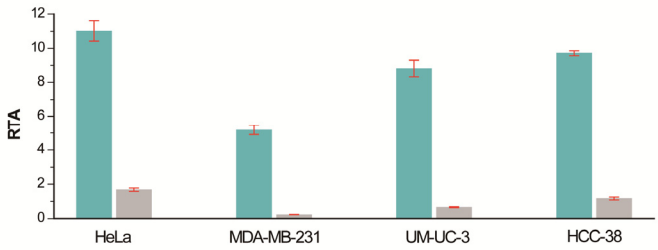

Fig. 3 The EXPIATR-AuNP assay (cyan) and the EXPIATR assay (grey) were performed respectively on cell extracts equivalent to 40 cancer cells of different cancer cell lines in the presence of a high concentration of foreign cell lysates (equivalent to 4,000 normal cells), and the resulting RTAs were quantified. Error bars indicate standard error of triplicate tests.

the assays tested on complex samples containing 4,000 normal cell lysates. The names of pure samples and complex samples are differentiated by suffix p (for pure samples) and c (for complex samples); the AuNP-based assays are differentiated from the traditional assays by adding primes to the names. Using the gel analysis, three main findings were revealed in regards to the roles of the normal cell lysates and AuNPs.

First the gel image verified that the abundance of foreign cell lysates in the complex samples would inhibit the specific amplifications. In the presence of 1,000 normal cell lysates, the traditional assays (Lane 1p vs 1c and Lane 2p vs 2c) show reduced intensities of the specific bands as compared with the product bands amplified from the pure samples; such inhibition effects were more significant when the interferant amount was increased to 4,000 normal cells (Lane 3p vs 3c, Lane 4p vs lane 4c, and Lane 5p vs 5c), *i.e.* the specific amplifications were significantly inhibited, while the non-specific products (based on the gel shift pattern of the negative control in Lane 6p) were increasingly amplified. This is especially true for the case in which a very small quantity of cancer cell extracts was present in the protein-rich background. For example in Lane 5c, the amplification products of the complex sample consisting of the extracts of 10 HeLa cancer cells and a 400-fold excess of foreign cell lysates were seriously predominated by the non-specific products, which produced much less the specific products as compared with the assay directly performed on the same amount of pure cancer cell extracts (Lane 5p).

Further we found that, whether performed on pure samples or complex samples, the AuNP-based assays showed improved specificity compared with the corresponding traditional assays. (1) For the detections of pure cancer cells, the traditional assays already showed good specificities without significant non-specific signals (Lanes 3p, 4p, and 5p), but the corresponding AuNP-based assays (Lanes 3p', 4p' and 5p') showed even greater specificity, in which the bands amplified from the longer telomerase extension products become substantially more. For example, in the presence of AuNPs, the detection of as few as 10 HeLa cancer cells gave a very clean gel image showing a ladder of specific bands (Lane 5p') longer than the products of the traditional assay (Lane 5p). Human telomerase is modestly processive *in vitro*, and with shorter telomerase products accumulating the longer telomerase extension products generally are less in quantity than the shorter ones by gel analysis.[38] As the outcomes of the assay can be influenced by both of the two reaction stages (the telomerase extension at 30 °C and the nucleic acid amplifications at 55 °C), the enhanced amplifications of longer telomerase extension products may be the result of two possibilities: the AuNP-based assays become more sensitive for the detection of template in low quantities or the telomerase processivity is enhanced by the addition of AuNPs. By employing a modified two-step protocol (Supporting Information, Fig. S5) to run the two stages independently, we proved that the enhancing effect of AuNPs is related to the enzymatic nucleic-acid-amplification process rather than the telomerization process. As a result, it was revealed that the addition of AuNPs could improve the sensitivity of the amplification reactions in the assay. (2) From the detections of the complex samples containing 1,000 normal cells, we found the inhibition effect of cell lyastes was fully eliminated in the AuNP-based assays. In the presence of AuNPs, the amplified products of the complex samples containing 1,000 foreign cells (Lanes 1c' and 2c') displayed almost identical gel patterns with those of the pure cancer cells (Lanes 1p' and 2p'), both of which showed enhanced amplifications of the longer telomerase extension products as compared with the traditional assays performed on the same amount of pure cancer cells (Lanes 1p and 2p). This result demonstrates that, more than simply overcoming the inhibition caused by the enriched cell lysates, the addition of AuNPs enhances the specificity of the assays to a new level. (3) The detections of complex samples containing 4,000 normal cells were investigated. The EXPIATR-AuNP assays also showed improved specificity compared with the traditional assay (Lane 3c' vs 3c, 4c' vs 4c, and 5c' vs 5c), though the AuNP effects became less significant (Lane 3c' vs 3p', 4c' vs 4p', and 5c' vs 5p'). This is accordant to the real-time results, *i.e.* with the addition of AuNPs the sensitivities of the assays performed on complex samples containing 4,000 normal cells were improved but could not reach the same level as the detections on the pure cancer cells. These results are due to the poor specificity of the assays induced by concentrated lysates from 4,000 normal cells. From the gel images of the negative controls, we learned that the non-specific reactions run in the presence of and in the absence of 4,000 foreign cells (Lane 6c and Lane 6p) produced different gel shift patterns, indicating that the outcome of the non-specific reaction would be affected by the enrichment of cell lysates. By the addition of AuNPs in the case absent of cell lysates, the non-specifically amplified products were significantly reduced (Lane 6p vs 6p'), which is in agreement with the results of the detections on pure cancer cells and on the complex samples containing foreign normal cells at relatively low concentrations; while in the presence of a high concentration of normal cell lysates, the suppression effect of AuNPs against the non-specific reactions appeared less significant (Lane 6c vs 6c').

Finally for all the assays, we found several faint high molecular-weight bands generated at the same positions, which

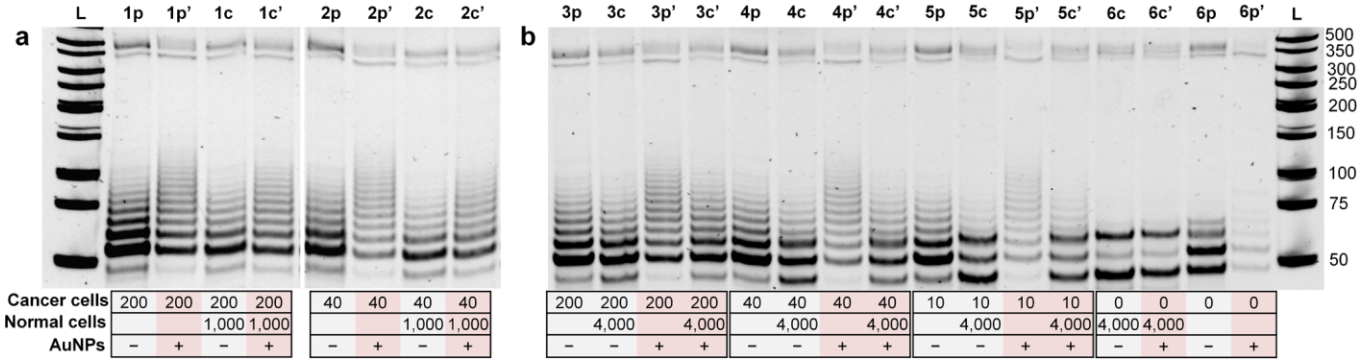

Fig. 4 Non-denaturating PAGE analysis of the traditional EXPIATR assay and the AuNP-based assay tested on the extracts of HeLa cancer cells, pure and in the presence of a high concentration of foreign cell lysates: (a) the assays tested on complex samples containing 1,000 normal cell lysates; (b) the assays tested on complex samples containing 4,000 normal cell lysates.

cannot be simply explained by the primer-based non-specific products due to their high molecular weights. These faint bands were believed to come from the DNA-independent non-specific synthesis conducted by the polymerase and nicking enzyme, which will be discussed in detail later. Taken together, the gel analysis revealed that the presence of concentrated cell lysates in the complex samples inhibited the specific amplifications and caused more non-specific reactions; while the addition of AuNPs would improve the sensitivity and specificity of the amplification reactions. These results inspired us to investigate in depth the role of AuNPs in the general process of the isothermal enzymatic amplifications, rather than limit the study to the EXPIATR assay.

To study the role of AuNPs in the process of nucleic acid amplifications, their effect on the non-specific reactions were examined first. The isothermal enzymatic amplification reaction used in the EXPIATR assay is a typical NE-SDA reaction using two enzymes, the *Bst* 2.0 Warmstart polymerase and the Nt.BspQI nicking endonuclease (NEase). According to previous reports, NE-SDA reactions might be accompanied by non-specific reactions of both primer artifacts and DNA-independent synthesis from the enzymes.[13] The primer artifacts are non-specifically synthesized from the primer mismatches, which generally exist both in PCR and other NAA methods. The other non-specific reaction is DNA-independent synthesis, which produces new DNA strands from dNTPs only by enzymes (the thermophilic polymerase and the NEase) without any templating or priming DNA strands, also called RE-pol DNA synthesis. Fig. 5a shows the non-specific reactions occurred at two extreme conditions, *i.e.* Lanes 1a and 1b were performed in the NEB3 buffer (the recommended buffer for the Nt.BspQI NEase), and Lanes 2a-2c were run in the isothermal amplification buffer (IA buffer, the recommended buffer for the *Bst* 2.0 polymerase). To study the DNA-independent non-specific reactions from the enzymes: Lanes 1a and 2a were run only in the presence of two enzymes and dNTPs (without any DNAs) in respective buffers. From the gel, we observed that the quick DNA-independent RE-pol synthesis occurred solely in the IA buffer (Lane 2a), and none was observed in the NEB3 buffer (Lane 1a). Second, in the presence of primers but no template, the non-template reactions were examined. Lane 1b with the NEB3 buffer showed the "primer-dimer" products only. Lane 2b with the IA buffer also showed the "primer-dimer" products, which had replaced the DNA-independent RE-pol synthesis, indicating the primer-related non-specific reactions are more favorable; however, certain faint bands were still produced at the position where the products of RE-pol synthesis would concentrate, indicating that a very small amount of RE-pol DNA synthesis still accompanied the primer-related reactions. Similar faint bands were also found in Fig. 4, suggesting the ubiquitous presence of the RE-pol DNA synthesis in NE-SDA reactions. When a high concentration of cell lysates (equivalent to 4,000 normal cells) was added to the non-template reaction in the IA buffer, Lane 2c showed that the RE-pol DNA synthesis became more significant (compared with the Lane 2b in absence of cell lysates). Finally, the influences of AuNPs on these non-specific reactions were explored. Lanes 2a′, 2b′, and 2c′ correspond to reactions of Lanes 2a, 2b, and 2c in the presence of AuNPs. In the absence of concentrated cell lysates, the addition of AuNPs reduced the non-specific reactions for both the amplified primer artifacts (Lane 2b') and the RE-pol DNA synthesis (Lane 2a'); while for the condition enriched with cell lysates, AuNPs only marginally affected the primer-artifact amplifications but significantly reduced the RE-pol DNA synthesis (Lane 2c'). These observations were in accordance with the data presented in Fig. 4, except that the RE-pol DNA synthesis became more significant in the IA buffer. Thus, the suppressing effect of AuNPs on the non-specific reactions, especially on RE-pol DNA synthesis, has been more elucidated.

Furthermore, we used the TPC8 template (the synthetic DNA target with eight telomere repeats will produce six laddered main products by the NE-SDA reaction) to examine the influence of AuNPs on the specific amplification reactions. One reason for using the TPC8 template is to exclude the telomerization step and study the AuNP effect on the NE-SDA reaction in a general way; another reason is that we can run the the amplifications over a wider range of target concentrations to collect more information. Fig. 5b shows PAGE analysis of the specific products amplified from the TPC8 template at two

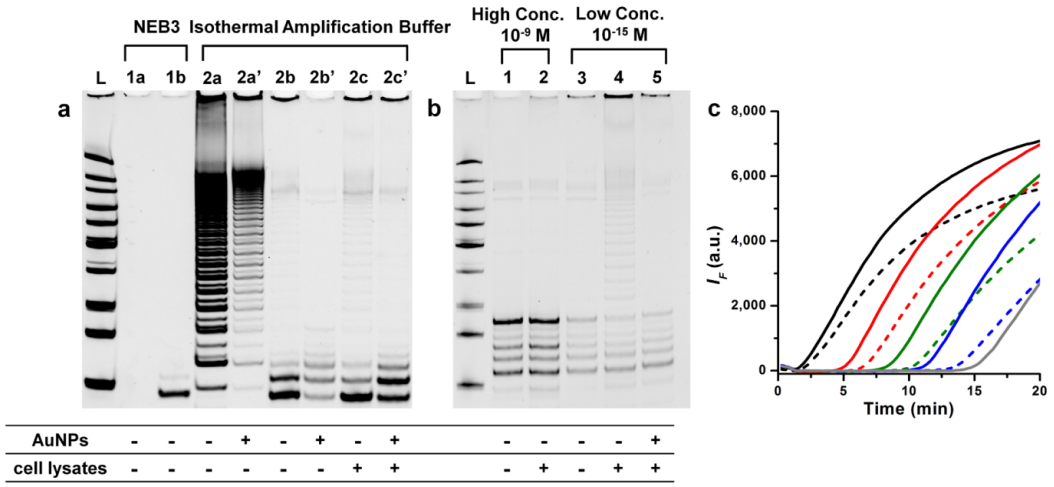

Fig. 5 Analysis of the influence of AuNPs on the NE-SDA reactions. (a) PAGE analysis of the non-specific reactions happened in NEB3 buffer and in isothermal amplification buffer: Lane 1a, Lane 2a, and Lane 2a' were DNA-independent synthesis; Lane 1b, Lane 2b, and Lane 2b' were non-template reactions; Lane 2c and 2c' were non-template reactions happened in the environment of a high concentration of cell lysates; (b) PAGE analysis of the specific reactions of the TPC8 template at different concentrations; (c) The real-time amplification curves corresponding to different concentrations of TPC8, pure (solid) and in the presence of a high concentration of cell lysates (dashed): 1×10^{-9} M (black), 1×10^{-11} M (red), 1×10^{-13} M (green), 1×10^{-15} M (blue), and negative control in the absence of TPC8 (grey).

different concentrations (all run in the IA buffer), one high concentration of 10^{-9} M and one low concentration of 10^{-15} M. In the case that a massive amount of TPC8 template (1×10^{-9} M) was targeted, very specifically amplified products were observed regardless of whether it was in the presence of cell lysates (Lane 1 and Lane 2); on the contrary, when a low concentration of TPC8 template (1×10^{-15} M) was amplified, the reaction in the presence of cell lysates (Lane 4) produced very clear non-specific signals of RE-pol DNA synthesis while the corresponding reaction absent of cell lysates did not (Lane 3). With the addition of AuNPs, the non-specific bands induced by cell lysates could be efficiently diminished as shown in Lane 5. This experiment further demonstrated AuNPs could reform the low-specificity of the nucleic-acid-amplification reactions caused by the presence of concentrated cell lysates.

As revealed by the studies on the real-time amplifications, we observed the cell lysates caused the inhibition of the amplification reactions in a target-concentration-dependent manner. Fig. 5c shows the amplifications with the concentrations of TPC8 ranging from 1×10^{-9} M to 1×10^{-15} M, in the absence of (solid lines) and in the presence of a high concentration of cell lysates (equivalent to 4,000 cells in each test) (dashed lines). As the TPC8 template concentration decreased, the inhibitory effect of the cell lysates to the amplification reactions became more apparent. The presence of cell lysates hardly affected the amplification of TPC8 when a high concentration was present, which implies that the main inhibitors from the cell lysates affected the amplification reaction by impairing the templates more than the enzymes. As cellular proteins which are prone to binding with DNA templates and making them unavailable for polymerases,[10,39] are believed to be essential inhibitors to NAA methods, we treated the normal cell lysates with proteinase K prior to the amplification process, and found that the negative effect of cell lysates on the amplification reactions was eliminated (Supporting Information, Fig. S6). However, the other pretreatments of the cell lysates by DNase or RNase were unsuccessful in removing the inhibitory effect (Supporting Information, Fig. S6), thus proving that the cellular proteins are the essential inhibitors to the amplification reactions. As the proteins in cell extracts might bind with ssDNAs and make the templates unavailable to polymerase,[39] a more significant inhibition effect was observed when fewer initial templates were present in the analyzed sample.

As it has been proven that AuNPs could enhance the low-specificity caused by the cell lysates, we examined whether the AuNPs work by removing the interference of cell lysates and as a result improve the reaction specificity indirectly, or rather the AuNPs work by enhancing the specificity of the amplification reaction in a fundamental way. The amplification reactions run using the wild-type *Bst* polymerase showed worse specificity than that by *Bst* 2.0 Warmstart polymerase. As shown in Fig. 6a, the amplification reactions using wild-type *Bst* polymerase intrinsically presented a low specificity. The amplification curves irregularly correlated with the starting concentrations of template (100-fold dilution series from 10^{-9} to 10^{-15} M); also some of the reactions showed poor reproducibility, *i.e.* the amplifications of templates at low concentrations were partially or completely inhibited. Studied by gel electrophoresis, the amplifications of 10^{-9} M TPC8 (Lane 1), 10^{-13} M TPC8 (Lane 2) and non-template control (Lane 3) were visualized. The image indicated that the specific amplifications were interfered by the non-specific reactions; the amplifications of 10^{-13} M TPC8 and non-template control were especially overwhelmed by the RE-pol DNA synthesis. The fluorescent probe used in this NE-SDA reaction is also acting as a primer, which is in a hairpin structure and modified with both a fluorophore and a signals in the specific reactions and the "primer-dimer" non-

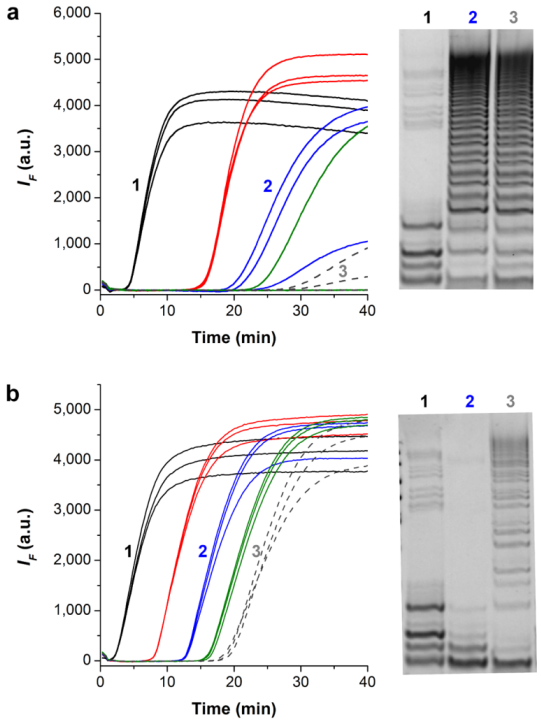

Fig. 6 The amplifications run by using the wild type *Bst* polymerase: different concentrations of TPC8 in the absence (a) and in the presence (b) of AuNPs: 1×10^{-9} M (black), 1×10^{-11} M (red), 1×10^{-13} M (blue), 1×10^{-15} M (green), and non-template control (gray); the gel images on the right corresponding to the products of the amplifications that were labelled with the same number.

specific reactions, but not in the RE-pol DNA synthesis because it is a DNA-independent reaction. As a result, when the RE-pol DNA synthesis overwhelmed the specific amplification in the case of low specificity, the fluorescence signals were inhibited. Accordingly, if probed by the SYBR Green I dye (a non-specific probe that will bind to any random dsDNA sequences) instead, only the amplifications of 10^{-9} and 10^{-11} M TPC8 could be distinguished from the non-template control, while the other amplification curves fell together (data not shown), since the products of RE-pol DNA synthesis could also probed by SYBR Green I dye. With the addition of AuNPs, the specificity of the amplification reactions conducted by the wild-type *Bst* polymerase could be significantly improved. As shown in Fig. 6b, the amplification curves evenly distribute along the concentrations of template (from 10^{-9} to 10^{-15} M). Furthermore, by the gel characterizations, we could see the non-specific RE-pol DNA synthesis had been greatly suppressed by the addition of AuNPs. In this example, even without cell lysates involved, the AuNPs could also suppress the non-specific reactions, and as a result, improve the specificity of the reactions, which indicated that the "AuNP effects" did not simply work only on cellular proteins but fundamentally on improving the amplification reactions.

The NEase enzymes used in the isothermal NE-SDA reaction will accelerate and intensify the non-specific reaction of the polymerase. As a result, in addition to the enhancing-effect of AuNPs on the specific amplifications, we further observed the suppressing effect of AuNPs on the non-specific reactions. Also, by studying the AuNP effect on an intrinsically low-specificity system caused by the less specific polymerase, we believe that AuNPs have the power to enhance and reform the NE-SDA reactions in a very fundamental way.

As AuNPs exhibit a characteristic surface plasmon band at ~520 nm, which is sensitive to the local environment of gold surface, UV-Vis spectroscopy is a powerful means to study the interaction between AuNPs and other biomolecules.[40] As seen in Fig. 7a, the UV-Vis absorption spectrum of the AuNPs solution dispersed in water displayed the typical plasmon peak at 514 nm (curve 1). If diluted by the reaction buffer (curve 2), the AuNPs themselves showed a red-shifted absorption at 610 nm, indicating an aggregated state; while in the presence of DNA reactants (primers, templates and dNTPs), the AuNPs showed absorption back to 514 nm (curve 3), changing its status from aggregation to colloid dispersion state. According to previous studies, citrate-capped AuNPs are stabilized by electrostatic repulsion. The high salt concentration in the amplification buffer will screen the repulsive interactions and cause colloid aggregation; while the ssDNAs will adsorb on the gold surface through favorable interactions between the bases and gold, making the AuNP surface more negatively charged and resistant to the bulk ionic strength induced by the buffer, and as a result, stabilize the particles against aggregation.[41]

The interactions between AuNPs and the reaction components were further studied using the one-by-one method as shown in Fig. 7b, *i.e.* the interactions of AuNPs with the components of the reaction mixture were studied gradually. (1) When AuNPs were absent in the reaction mixture (curve 4), no significant absorption could be observed. (2) When reactant enzymes (*Bst* 2.0 WarmStart polymerase and Nt.BspQI NEase) were eliminated from the reaction mixture (curve 3), as discussed above, the ssDNAs would stabilize the AuNPs from aggregation and result in a dispersion state. (3) Alternatively, when DNA reactants were eliminated from the reaction mixture (curve 5), the absorption band of AuNPs became broader and showed a peak at 530 nm. The red-shift of the absorption under this condition was not as pronounced as that corresponding to the aggregation state and parts of the absorption still came from the un-aggregated state, indicating that the enzymes probably induced agglomeration of the AuNPs rather than aggregation.[42-44] (4) When all the reactants were involved (curve 6), a further red-shifted and broader absorption was observed compared with the agglomeration state induced by the enzymes alone. It indicated that the enzymes might not remove the citrate ligands from the gold surface but rather form self-assemblies with the particles through electrostatic interactions between the positively charged amino groups of proteins and the negatively charged citrate groups.[43] Therefore, in the presence of DNA reactants, a higher charge density would be generated on the AuNP surface and enhance the electrostatic attraction to the enzymes; thus a more red-shifted absorption was observed when the reactant DNAs and enzymes coexisted in the system. Subsequently, the influences of cell lysates on the reaction system were studied as shown in Fig. 7c. The absorption spectrum of AuNPs in the reaction mixture with cell lysates (curve 7) was very similar to that without cell lysates, except

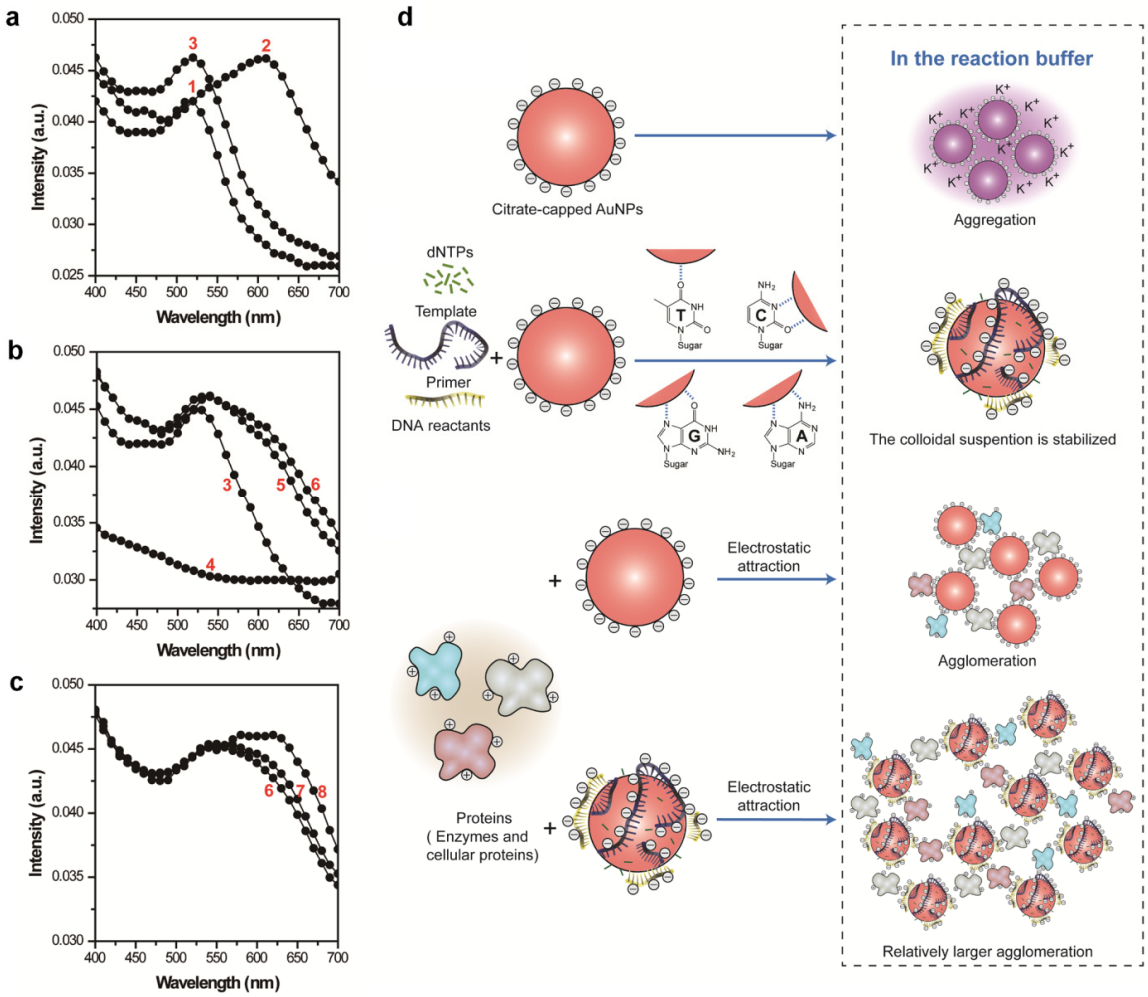

Fig. 7 UV–Vis spectroscopic analysis of the interactions between AuNPs and the reaction components. (a): diluted water solution of AuNPs (curve 1), AuNPs in the reaction buffer (curve 2), and AuNPs in the reaction buffer mixed with DNA reactants (curve 3); (b): a reaction mixture without AuNPs (curve 4), a reaction mixture with AuNPs in the absence of enzymes (curve 3), a reaction mixture with AuNPs in the absence of DNA reactants (curve 5), and a full reaction mixture with AuNPs (curve 6); (c): a full reaction mixture with AuNPs (curve 6), a full reaction mixture with AuNPs in the presence a high concentration of cell lysates (curve 7), and a reaction mixture in the presence of AuNPs and with the enzymes replaced by a high concentration of cell lysates (curve 8); (d) Schematics illustrating the interactions between AuNPs and the reaction components.

being a little broader. When the two enzymes were eliminated from the reaction mixture and replaced by cell lysates (curve 8), the resultant spectrum also showed a broad absorption band with a red-shifted peak; This indicates that cell lysates had similar interactions with AuNPs to the enzymes, which is expected given that both are protein molecules. However, the red-shift of the absorption that the cell lysates induced was more significant than that of the enzymes, suggesting the cell lysates might induce a different agglomeration state of AuNPs. Furthermore, we found that the absorption band of the reaction mixture in the presence of both the enzymes and the cell lysates (curve 7) fell between the absorptions of reaction mixtures containing solely the enzymes or solely the cell lysates (curve 6 and curve 8), but was more similar to that of the enzymes-only mixture (curve 6). This suggests that when both the enzymes and the cell lysates were added to the reaction mixture, AuNPs would show a stronger affinity to the enzymes than the cellular proteins. The proposed interactions of AuNPs with the components of the assay were illustrated in Fig. 7d.

According to the results of the UV-Vis spectroscopic studies, we can understand more about the AuNP effect. First since AuNPs have a high surface area / volume ratio and strongly interact with all the reaction components, the addition of AuNPs could significantly change the distribution of reaction components in the amplification system, which may help us to understand how such a small amount of AuNPs could affect the specificity and sensitivity of the amplification reactions. Also, the strong interactions between AuNPs and the reaction components reveal the concentration of AuNPs to be very critical, since a certain amount of AuNPs ($\leq$ 0.4 nM for this case) can enhance the specificity of the amplification reactions by raising the probability of dynamical contacts between templates and enzymes, but too many AuNPs will inhibit the reactions since the excessive binding of the enzymes to AuNPs

would contrarily decrease activities of the enzymes.[31] Meanwhile, we found that 0.4 nM AuNPs also showed negligible quenching effect of the fluorescence probe (Supporting Information, Fig. S7), further indicating it as a good concentration for the NE-SDA reactions. Last of all, the situation is more complicated for the EXPIATR assay performed on complex samples. As the UV-Vis spectroscopic study demonstrated that both cell lysates and enzymes have strong affinity for AuNPs, the cellular proteins will partially bind to the nanoparticle surfaces and compete with the enzymes. Thus, on one hand, the cell lysates will counteract the effect of AuNPs, *i.e.* [AuNPs] ≤ 0.4 nM only marginally suppressed the primer-artifact amplification and showed noticeably less improvements in the specific amplification (as we discussed in Fig. 4) in the assays performed on complex samples containing 4000 normal cells; on the other hand, the cell lysates will protect enzymes from excessive binding to AuNPs. As a result, the addition of more AuNPs (> 0.4 nM) would not immediately inhibit the specific reactions, but further diminish the influence from cell lysates of complex samples. However, [AuNPs] > 0.4 nM is still not desirable for EXPIATR assay, as more AuNPs will induce a false-positive problem. For example, with the addition of 0.5 nM AuNPs, the assay performed on a large amount of normal cells (enriched cellular proteins will protect the enzymes from excessive binding) exhibited a higher amplification rate than the non-template control in the absence of cell lysates, resulting in a false-positive (Supporting Information, Fig. S8). With these considerations, 0.4 nM AuNPs, the concentration before inhibiting the amplification of pure samples, is considered optimal for the EXPIATR assay. Although the assay with 0.4 nM AuNPs could not achieve the maximum effect of AuNPs on eliminating the inhibition induced by 4,000 foreign normal cells, the sensitivity of the detections of telomerase activity in complex samples was still significantly improved, over five-fold as compared with the traditional assay; at the same time, detections on normal cells could also accurately show negative telomerase activity.

Conclusion

In this paper, we revealed that AuNPs are powerful additives to the isothermal NE-SDA reaction, which uses strategy distinct from PCR. Also, no matter the source of the low-specificity of the NE-SDA reaction, external (cell lysates in complex samples) or internal (low specificity of polymerase), AuNPs showed a similar power in improving the specificity of the reactions. These results imply that AuNPs improve the process of nucleic acid amplifications in a very fundamental way. It has been reported that nanomaterials (such as citrate-capped AuNPs, single-walled carbon nanotubes, and modified quantum dots) could enhance the structural stability and maintain the bioactivity of enzymes.[45-48] According to our observations, this is also the most likely mechanism of the AuNP effect in this study. There are some other possible mechanisms of the AuNP effects which may also explain some of our results. For example, it has been believed that the AuNPs may resemble the function of the single-strand-binding protein in the course of nucleic acid amplification,[49] which selectively binds to ssDNA rather than dsDNA,[50] and prevents the amplifications of primer mismatches. Moreover, by the UV-Vis spectroscopic study, we observed the "concentrating effect" of AuNPs,[47] *i.e.* all the reaction components were attracted towards the charged nanoparticles. The aggregates of all the necessary components for the amplification reaction surround the AuNPs, resulting in many small reaction centers, raising the probability of dynamical contacts between templates and enzymes, and thus, enhancing the sensitivity of the amplification of a low template quantity within a high concentration of background cell lysates. In conclusion, our investigations demonstrated that AuNPs have powers in enhancing the specificity of isothermal nucleic acid amplification reactions. Such AuNP effects provide efficient ways to improve the detection sensitivity of the EXPIATR assay performed on complex samples containing enriched normal cell lysates; otherwise, enriched cell lysates would impair the specificity of nucleic acid reactions and cause troubles for the related assays in clinical applications. A proper concentration is important to appreciate the power of the AuNPs, which has to be carefully optimized to ensure the amount of AuNPs will not affect the amplification significantly in the absence of cell lysates. Given the importance of the strategy of using nicking enzymes and DNA polymerases to develop isothermal NAA methods, this study will be significant by providing an efficient method to enhance the reliability for related isothermal assays in clinical applications.

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Notes and references

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