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ARTICLE

Dual-recognition-based determination of ctDNA via the clamping function of peptide nucleic acid and terminal protection of small-molecule-linked DNA

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A new dual-recognition fluorescent biosensor for circulating tumor DNA (ctDNA) detection has been developed, which combines the clamping function of peptide nucleic acid (PNA) and terminal protection of small-molecule-linked DNA (TPSMLD). Taking the tumor-specific E542K mutation and methylation of PIK3CA gene as the target ctDNA, a low detection limit of 0.3161 pM ctDNA is obtained with a good selectivity. This study not only offers a sensitive, selective and accurate ctDNA detection method, but also can be used to detect the target in complex biological samples.

1 Introduction

Circulating tumor DNA (ctDNA) is small DNA fragments that is released by circulating tumor cells.[1] CtDNA has been recognized as a promising biomarker in cancer diagnosis and monitoring because it carries tumor-related alterations, such as point mutations and DNA methylation. However, the accurate detection of ctDNA is challenging due to the low concentration of ctDNA.[2] The existing methods able to monitor ctDNA rely on polymerase chain reaction (PCR),[3] DNA sequencing[4] and bisulfite-based methods.[5] PCR is complex in steps and susceptible to interference from the biological samples, resulting in false positive/negative signals.[6] Besides, PCR is usually not suitable for detecting point mutations.[7] Compared with PCR, DNA sequencing is an excellent approach for ctDNA mutation analysis, but it requires expensive equipment and long analysis time, which limits its application.[8] The bisulfite-based methods are widely and successfully used in DNA methylation assays.[9] However, DNA degradation may be exacerbated by the acidic and high temperature conditions during bisulfite conversion. More importantly, all of the above methods focus on the identification of a single biomarker (gene mutation or methylation), which makes it difficult to ensure the accuracy of ctDNA detection. Therefore, it's important to develop a highly sensitive platform for ctDNA detection through dual biomarkers recognition of genetic mutation and methylation in one step.

Peptide nucleic acid (PNA) as a versatile probe has been successfully used to detect and screen for single base-pair gene variants in a 100-fold excess of wildtype background. PNA can bind double-stranded DNA (dsDNA) with high affinity by the mechanism of displacement.[10] The PNA/dsDNA hybrid is stable due to the lack of electrostatic repulsion between the neutrally charged PNA and the negatively charged phosphate backbone of DNA molecules. Particularly, PNA/DNA mismatch is more unstable than a mismatch in a DNA/DNA,[11] for example, a single mismatch of PNA/DNA could reduce the melting temperature by 15 °C, while a single mismatch of DNA/DNA could only reduce the melting temperature by 4 °C.[12] Therefore, PNA molecule can be an ideal probe for detecting point mutation and can eliminate false positive readings under thermal control conditions.

The binding of small-molecule-linked DNA to target protein may provide an ideal method for DNA methylation analysis. The principle of this method is that, small molecule-linked single-stranded DNA (ssDNA) can be protected from exonuclease I (Exo I) digestion when the small molecule is bound to its protein target.[13] This finding is called terminal protection of small-molecule-linked DNA (TPSMLD), which has encouraged many researchers to develop different analytical strategies for detecting the small molecule-protein interactions.[14] Although these TPSMLD-based methods present distinct advantages, they also have their own limitations, such as low sensitivity. Exonuclease III (Exo III)-assisted DNA recycling amplification has been widely used to improve sensitivity due to its excellent universality and practicability.[15] Therefore, the minor changes of DNA methylation levels in ctDNA can be quantified by introducing Exo III-assisted DNA recycling into TPSMLD assay.

In order to improve the accuracy of ctDNA detection, a sensitive platform for dual biomarker recognition of double stranded ctDNA (ds-ctDNA) was developed by combining PNA-assisted TPSMLD and Exo III-aided DNA recycling. In

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this system, the PNAs on the magnetic microparticles are employed to clamp the mutated sequence, and TPSMLD combined with Exo III-aided DNA recycling is used for methylation analysis. The concentration of ds-ctDNA is determined by the enhanced fluorescence signal, which is produced only when the gene mutation and methylation are recognized at the same time.

2 Experimental section

2.1 Chemicals and reagents

Streptavidin-modified magnetic microparticles (MMPs) (10 mg mL^{-1}) were purchased from Invitrogen Corporation (USA). 5-methylcytosine monoclonal antibody (anti-5mC) was purchased from Epigentek (USA). Exonuclease I (Exo I) and Exonuclease III (Exo III) were purchased from Takara Biotechnology (Dalian) Co., Ltd. Sodium chloride (NaCl) and Magnesium chloride ($MgCl_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical reagent grade. Peptide nucleic acid (PNA) for hot-spot E542K was synthesized and purified by Tahepna Biotechnology Co., Ltd (Hangzhou, China). DNA oligonucleotides were synthesized and purified by Sangon Biotechnology Co., Ltd (Shanghai, China). The sequences of PNA and oligonucleotides were as follows (from 5' to 3'):
PNA: TCAGTGATTTTAGAG-Lys (biotin);
Mutated sequence in E542K-ds-ctDNA:
AGATCCTCTCTCTAAAATCACTGAGCAGGAGAAAGATTTTCTATGGAGTCACAGA;
Complementary sequence in E542K-ds-ctDNA:
TCTGTGACTCCATAGAAAATCTTTCTCCTGCmTCAGTGATTTTAGAGAGAGGATCT;
E542K-MB:
FAM-ACTCCATGAGAAAGATTTTCTATGGAGT-BHQ1-CACAGA;
Wildtype sequence in E542K-ds-ncDNA:
AGATCCTCTCTCTGAAATCACTGAGCAGGAGAAAGATTTTCTATGGAGTCACAGA;
Complementary sequence in E542K-ds-ncDNA:
TCTGTGACTCCATAGAAAATCTTTCTCCTGCTCAGTGATTTCAGAGAGAGGATCT;
Mutated sequence in E545K-ds-ctDNA:
TCTCTGAAATCACTAAGCAGGAGAAAGATTTTCTATGGAGTCACAGACACTATTGTG;
Complementary sequence in E545K-ds-ctDNA:
CACAATAGTGTCTGTGACTCCATAGAAAATCTTTCTCCTGCmTTAGTGATTTCAGAGA;

The E542K-MB was heated to 95 °C for 5 min and then allowed to cool to room temperature for 1 h before use.

2.2 Instrumentation

Fluorimetric spectra were obtained with a F-4600 FL Spectrophotometer (Hitachi, Japan) equipped with a 150 W xenon lamp (Ushio Inc, Japan). Ultrapure water obtained from a Millipore water purification system (Billerica, MA, USA) was used in all of the assays. The human serum sample was supplied by Union Hospital of Huazhong University of Science and Technology.

2.3 Preparation of PNA-functionalized magnetic microparticles

Streptavidin-coated magnetic microparticles (MMPs) (250 μL, 10 mg mL^{-1}) suspension were washed three times with 1 mL binding buffer (10 mM PBS, 150 mM NaCl, pH 7.40) and resuspended in 230 μL binding buffer. Then, 20 μL biotin-modified PNAs (26.2 μM) in ultra-pure water were added in the MMPs suspension and incubated at room temperature with gentle shaking for 30 min. After incubation, the excess PNAs were removed by washing three times with binding buffer and the PNA-MMPs resuspended in 250 μL binding buffer for further use.

2.4 Optimization of the concentration of PNA-MMPs

Firstly, 30 μL E542K-ds-ctDNA (1 nM) and 0, 1, 5, 10, 15, 20 μL PNA-MMPs (10 mg mL^{-1}) solutions were mixed. Then the mixture was diluted with ultra-pure water to 300 μL and incubated with gentle shaking for 30 min at 62 °C. After washing with 10 mM PBS-Tween buffer (150 mM NaCl, 0.1% Tween 20, pH 7.40), 300 μL 5-methylcytosine monoclonal antibody (anti-5mC) (2.25×10^{-2} μg μL^{-1}) solution was added and incubated for 1 h at room temperature. Subsequently, the mixture was washed three times with PBS-Tween buffer. Exo I (10 U) and Exo III (25 U) were added to the above mixture, diluted with 10 mM PBS buffer (150 mM NaCl, 5 mM $MgCl_2$, pH 7.40) to 200 μL and incubated for 1 h at 37 °C. The supernatant was obtained after heating for 10 min at 95 °C. Next, 30 μL E542K-MB (1 μM) and 40 U Exo III were added to the supernatant, diluted with 10 mM PBS buffer (150 mM NaCl, 5 mM $MgCl_2$, pH 7.40) to 300 μL and incubated for 90 min at 37 °C. Finally, the fluorescence intensity was measured in the range 505 nm ~ 650 nm with excitation at 480 nm.

2.5 Optimization of the amount and reaction time of Exo III

To optimize the concentration of Exo III, 30 μL complementary sequence in E542K-ds-ctDNA (1 nM) and 30 μL anti-5mC (0.225 μg μL^{-1}) solutions were mixed and incubated for 1 h at room temperature. Then 30 μL E542K-MB (1 μM) and different amounts of Exo III (0, 5, 10, 20, 40, 60, 80 U) were added to the above mixture, diluted with 10 mM PBS buffer (150 mM NaCl, 5 mM $MgCl_2$, pH 7.40) to 300 μL and incubated for 90 min at 37 °C. After incubation, the fluorescence intensity of the solution was measured in the range 505 nm ~ 650 nm with excitation at 480 nm.

To optimize the reaction time of Exo III, 30 μL complementary sequence in E542K-ds-ctDNA (1 nM) and 30 μL anti-5mC (0.225 μg μL^{-1}) solutions were mixed and incubated for 1 h at room temperature. Then 30 μL E542K-MB (1 μM) and 40 U Exo III were added to the above mixture, diluted with 10 mM PBS buffer (150 mM NaCl, 5 mM $MgCl_2$, pH 7.40) to 300 μL and incubated for different time (0, 30, 40, 50, 60, 90, 120 min) at 37 °C. After incubation, the fluorescence intensity of the solution was measured in the range 505 nm ~ 650 nm with excitation at 480 nm.

2.6 Procedure for E542K-ds-ctDNA detection

The PNA-MMPs (10 μL) were incubated with 300 μL different concentrations of E542K-ds-ctDNA (0, 0.1, 0.3, 1, 2, 5, 10 × 10^{-11} M) for 30 min at 62 °C. After that, the mixture was washed three times with PBS-Tween buffer. Then, 300 μL anti-5mC (2.25 × 10^{-2} μg μL^{-1}) solution was added and incubated for 1 h at room temperature. Subsequently, the mixture was washed three times with PBS-Tween buffer. Exo I (10 U) and Exo III (25 U) were added to the above mixture, diluted with 10 mM PBS buffer (150 mM NaCl, 5 mM $MgCl_2$, pH 7.40) to 200 μL and incubated for 1 h at 37 °C. The supernatant was obtained after heating for 10 min at 95 °C. After rinsing with PBS-Tween buffer, 30 μL E542K-MB (1 μM) and 40 U Exo III were added to the supernatant, diluted with 10 mM PBS buffer (150 mM NaCl, 5 mM $MgCl_2$, pH 7.40) to 300 μL and incubated for 90 min at 37 °C. Finally, the fluorescence intensity was measured in the range 505 nm ~ 650 nm with excitation at 480 nm.

2.7 Performance of E542K-ds-ctDNA detection in serum

Different concentrations of E542K-ds-ctDNA were diluted with human serum. The remaining steps for E542K-ds-ctDNA detection in serum were almost as same as 2.6.

3 Results and discussion

3.1 The principle of the dual-recognition method for ctDNA detection

As shown in Fig. 1, PNA probe was used for recognition of genetic mutation in ctDNA. The PNA was designed perfectly complementary to the mutated sequence of ctDNA, and had a mismatch for normal circulating DNA (ncDNA). Thus, there is a big difference (11 °C) in melting temperature between PNA/ctDNA and PNA/ncDNA. For capturing the target, PNA-MMPs and E542K-ds-ctDNA were incubated at 62 °C. At this temperature, the target ctDNA could hybridize with PNA normally, while most E542K-ds-ncDNA were melted from PNA-MMPs because the melting temperature of ncDNA was 54 °C.[11b] Therefore, the denatured E542K-ds-ncDNA molecules were removed at 62 °C. Then, methylation in ctDNA was recognized by specifically binding of anti-5mC and methylated CpG sites. After incubation with exonuclease, the mutated strand of E542K-ds-ctDNA was degraded from its blunt 3'-end by Exo III, and the complementary strand of E542K-ds-ctDNA was protected from digestion due to the binding of anti-5mC and methylation. Subsequently, the complementary strand hybridized with molecular beacons (E542K-MBs) and opened its

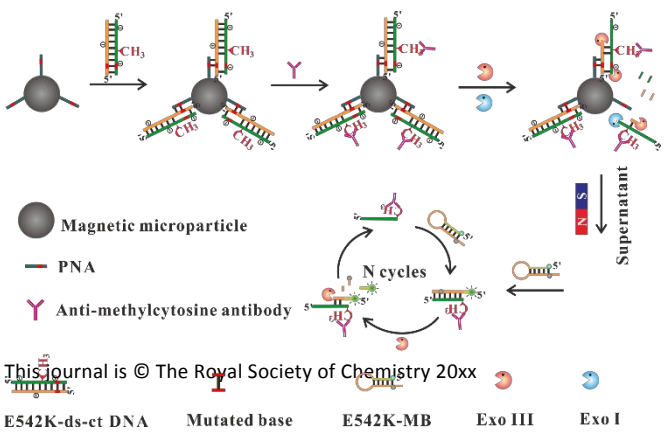

Fig. 1 The mechanism of the dual-recognition fluorescence biosensor for E542K-ds-ctDNA.

stem–loop structure. Meanwhile, the blunt 3'-end of the E542K-MBs formed by hybridization was degraded by Exo III. Consequently, the remaining complementary strand was rolled into the next signal generation cycle, making more fluorophore keep away from the quencher and send out the strong fluorescence signal.

3.2 The feasibly of this biosensor for dual-recognition of ctDNA

To verify the feasibility of this method, the fluorescence under different conditions were investigated. As illustrated in Fig. 2, in the presence of Exo III and E542K-MBs (curve a), no significant change in fluorescence was observed compared with the weak fluorescence of E542K-MBs (curve b), indicating the Exo III had scarce influence on the E542K-MBs. In the absence of anti-5mC (curve c), a weak fluorescent signal was exhibited. This was due to fact that the target E542K-ds-ctDNA was digested by exonuclease and couldn't perform signal amplification. In addition, the fluorescence increased in the presence of the target ctDNA, anti-5mC, Exo I + Exo III, and E542K-MBs (curve d). It indicates that the fluorescence signal could enhance only when the dual recognition of gene mutation and methylation were present. When the target ctDNA, anti-5mC, Exo I + Exo III, Exo III and MBs were present (curve e), the fluorescence signal was significantly enhanced due to the Exo III-assisted recycling amplification. Therefore, dual-recognition for ctDNA detection via Exo III-aided DNA recycling amplification is feasible.

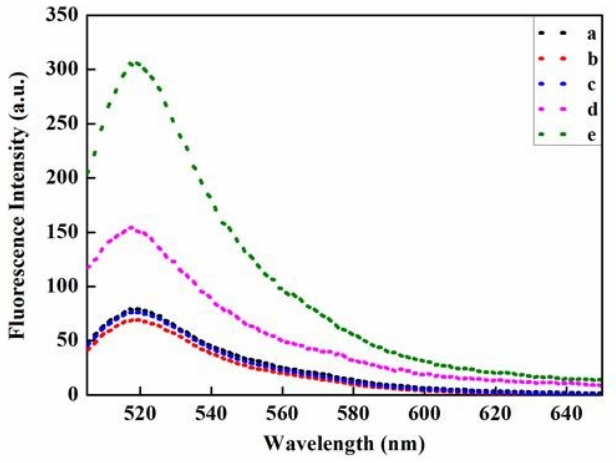

Fig. 2 Fluorescence spectra of the supernatant under different condition: a) Exo III+E542K-MBs; b) E542K-MBs; c) ctDNA+(Exo I+Exo III)+Exo III+E542K-MBs; d) ctDNA+anti-5mC+(Exo I+Exo III)+E542K-MBs; e) ctDNA+anti-5mC+(Exo I+Exo III)+Exo III+E542K-MBs. PNA-MMPs, 0.33 mg mL^{-1}; E542K-ds-ctDNA, 2 × 10^{-11} M; anti-5mC, 2.25 × 10^{-2} μg μL^{-1}; Exo III, 40 U; E542K-MB, 0.1 μM.

3.3 Optimization of experimental conditions

In order to obtain obvious fluorescence signals, the reaction conditions were optimized. Fig. S1 showed the effect of PNA-MMPs concentration on fluorescence intensity. The fluorescence intensity increased with the increasing

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concentration of PNA-MMPs, and reached to a plateau when PNA-MMPs concentration was up to 0.33 mg mL^{-1}. Thus, 0.33 mg mL^{-1} PNA-MMPs was chosen as the optimal concentration. Besides, the digestion procedures of Exo I and Exo III played vital roles in the subsequent methylation recognition. So, the amounts of Exo I and Exo III were optimized. As shown in Fig. S2, the optimal amounts of Exo I and Exo III in digestion procedures were 10 U and 25 U, respectively. Because Exo III directly affected to signal amplification reaction, the amount of Exo III used in signal amplification step was also optimized. As shown in Fig. S3, the fluorescence intensity increased progressively from 5 to 40 U and reached a peak value at 40 U. Further, the reaction time of Exo III was investigated in the presence of 40 U Exo III (Fig. S4). It was observed that fluorescence intensity increased rapidly in 90 min and then reached a plateau due to the complete degrading of dsDNA by Exo III, which also meant the end of Exo III-aided DNA recycling amplification. Therefore, 40 U Exo III for 90 min was taken as the optimized condition for the signal amplification.

3.4 ctDNA detection based on dual-recognition platform

Under the optimized conditions, the dual-recognition platform for E542K-ds-ctDNA detection was carried out. The fluorescence emission spectra of the biosensor in the presence of different concentrations of E542K-ds-ctDNA were displayed in

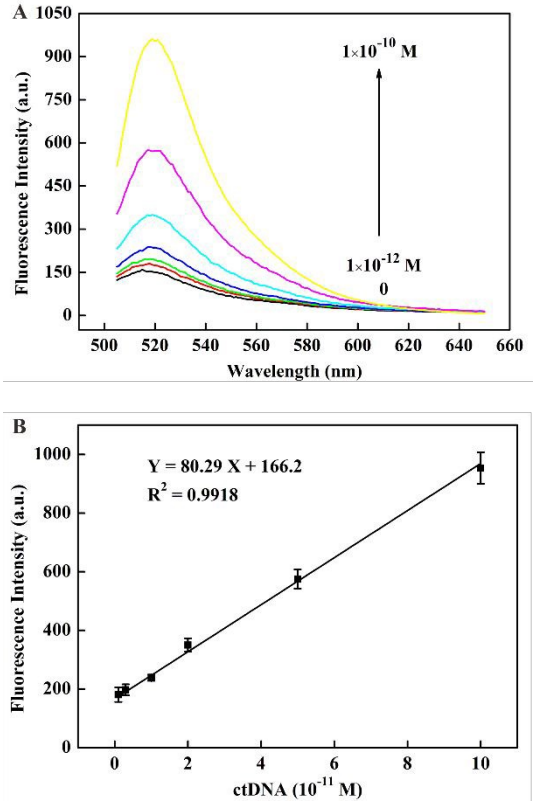

Fig. 3 Fluorescence spectra of the dual-recognition fluorescence biosensor upon the addition of increasing concentration of E542K-ds-ctDNA. (B) Calibration curve for E542K-ds-ctDNA detection. PNA-MMPs, 0.33 mg mL^{-1}; anti-5mC, 2.25 × 10^{-2} μg μL^{-1}; Exo III, 40 U; E542K-MB, 0.1 μM.

Fig. 3. The fluorescence intensity was increased with the increasing E542K-ds-ctDNA concentration. A good linear relationship has been obtained between the fluorescence intensity at 520 nm and the E542K-ds-ctDNA concentration in the range of 1 × 10^{-12} to 1 × 10^{-10} M with linear equation y = 80.29x + 166.2, where y is the fluorescence intensity at 520 nm and x (R^2 = 0.9918) is the concentration of E542K-ds-ctDNA. The limit of detection (LOD) is calculated to be 0.3161 pM based on the noise level of 3σ (where σ represents the relative standard deviation of the blank solution, n = 7), which is comparable to or exceeded the reported methods with the recognition of one biomarker (Table 1).[16] Compared with electrochemical assays, [6,17] this method could not only effectively avoid the complicated modification of electrodes, but also provided comparable sensitivity with dual recognition of biomarkers. Thus, this developed biosensor could be applied to quantification of ctDNA with high accuracy and sensitivity.

Table 1. Comparison of the reported methods for ctDNA detection

Method	Recognition of biomarkers	LOD	References
Fluorescence	genetic mutation	0.85 nM	16a
Colorimetric	genetic mutation	0.1 pM	16b
Colorimetric	genetic mutation	0.32 pM	16c
Electrochemistry	genetic mutation	3 pM	6
Electrochemistry	genetic mutation	0.65 nM	17
Fluorescence	genetic mutation and methylation	0.3161 pM	This work

3.5 The specificity of the dual-recognition E542K-ds-ctDNA biosensor

The specificity of this sensor was evaluated by incubating with E542K-ds-ncDNA, E545K-ds-ctDNA and target E542K-ds- ctDNA. As depicted in Fig. 4, there was an obvious fluorescence

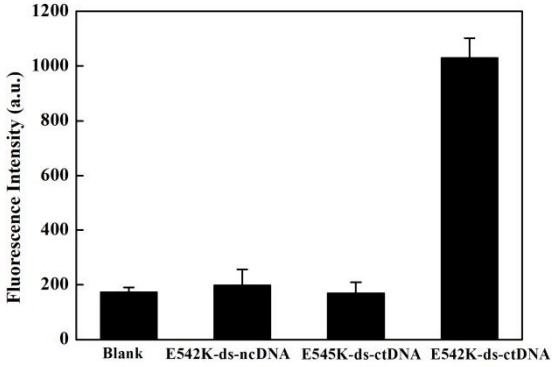

Fig. 4 Bar chart of the fluorescent intensities in the presence of different DNA sequences. Blank control (without E542K-ds-ctDNA),

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E542K-ds-ncDNA (1×10^{-8} M), E545K-ds-ctDNA (1×10^{-8} M) and target E542K-ds-ctDNA (1×10^{-10} M). PNA-MMPs, 0.33 mg mL^{-1}; anti-5mC, 2.25×10^{-2} μg $μL^{-1}$; Exo III, 40 U; E542K-MB, 0.1 μM.

increase in the presence of target E542K-ds-ctDNA, while the fluorescence was much weaker in the presence of E542K-ds-ncDNA and methylation E545K-ds-ctDNA. This was attributed to the dual specific binding between PNA and ctDNA, anti-5mC and methylated CpG sites. These results also indicated that the fluorescence signal could only be observed when both the recognition of gene mutation and methylation were present. Thus, this dual recognition biosensor could be applied to the detection of E542K-ds-ctDNA with high specificity.

3.6 E542K-ds-ctDNA detection in serum

To prove the potential of this biosensor in clinical applications, a recovery test of E542K-ds-ctDNA was carried out in human serum. Different concentrations of E542K-ds-ctDNA were diluted in human serum and the detection process was the same as in buffer. The recoveries were calculated to be 92.53, 103.7, 106.5 and 102.8%, respectively, with a relative standard deviation (RSD) from 1.84% to 5.16% (Table S1), exhibiting the adequate accuracy and good reproducibility of this present strategy. The results also indicated that this biosensor had a potential application in clinical detection of the E542K-ds-ctDNA.

4 Conclusions

In conclusion, we have successfully developed a novel and sensitive dual-recognition fluorescent biosensor for ctDNA detection. On one hand, with the introduction of PNA, this biosensor could distinguish the mutated ctDNA from wildtype DNAs. On the other hand, methylation in ctDNA could be analyzed by introducing Exo III-assisted DNA recycling into TPSMLD. Taking the E542K-ds-ctDNA of PIK3CA gene as the target ctDNA, E542K-ds-ctDNA is detected in a range of 1×10^{-12} to 1×10^{-10} M with a low LOD (0.3161 pM) and high selectivity. Further, this approach can be useful for multiplexed ctDNA detection with appropriate design of PNA and the commercial differently-labelled molecular beacons, showing great potential in high-throughput ctDNA analysis.

Conflicts of interest

There are no conflicts to declare.

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