Electronic Supplementary Information

A new mode for highly sensitive and specific detection of DNA based on exonuclease III-assisted target recycling amplification and mismatched catalytic hairpin assembly

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EXPERIMENTAL SECTION

Materials and Reagents. DNA oligonucleotides were synthesized and purified by Sangon Inc (Shanghai,China). Their sequences are listed in Table S1. 6-Mercapto-1-hexanol (MCH), streptavidin-alkaline phosphatase (ST-AP), α-naphthyl phosphate (α-NP), bovine serum albumin (BSA) and salmon sperm DNA were purchased from Sigma-Aldrich (USA). Exonuclease III (Exo III) was obtained from New England Biolabs (Beijing, China). 20 bp DNA Ladder Marker and MiniBEST Universal Genomic DNA Extraction Kit Ver.5.0 were purchased from Takara (Dalian, China). All other reagents were of analytical grade, and Millipore-Q water (≥18 MΩ) was used in all experiments.

Table S1. Sequences of the used oligonucleotides (in 5′ to 3′ direction)

Oligonucleotide	Sequence (5′ - 3′)
Capture probe	SH-$(CH_2)_6$-TTTTGAGTAGAGTCTGA
Target oligonucleotide	CTAGTGATTAGCTTATCAGAACCTGT
Hairpin probe 0 (H0)	TAGCTTATCAGACTGATGTTGACCATGTGTACATCT GATAAGCTAATCACTAG
Hairpin probe 1 (H1)	TCAACATCAGTCTGATAAGCTACCATGTGTAGATAG CTTATCAGACTCTACTCA
Hairpin probe 2 (H2)	TAAGCTATCTACACATGGTAGCTTATCAGAGACCAT GTGTAGATTT-biotin
Single-base-mismatched oligonucleotide	CTAGTGATTAGCTTATCATAACCTGT
Two-base-mismatched oligonucleotide	CTAGTGATTAACTTATCATAACCTGT
Non-complementary oligonucleotide	TGTCCATAATCTACTCATACAATTCA

Apparatus. All electrochemical measurements were performed on a CHI 660D electrochemical workstation (Shanghai Chenhua Instruments Co. Ltd., China) with a conventional three electrode system composed of platinum wire as auxiliary, Ag/AgCl electrode as reference, and a 3-mm-diameter gold electrode as working electrode. Gel images were recorded on an imaging system (Bio-Rad Laboratories, USA).

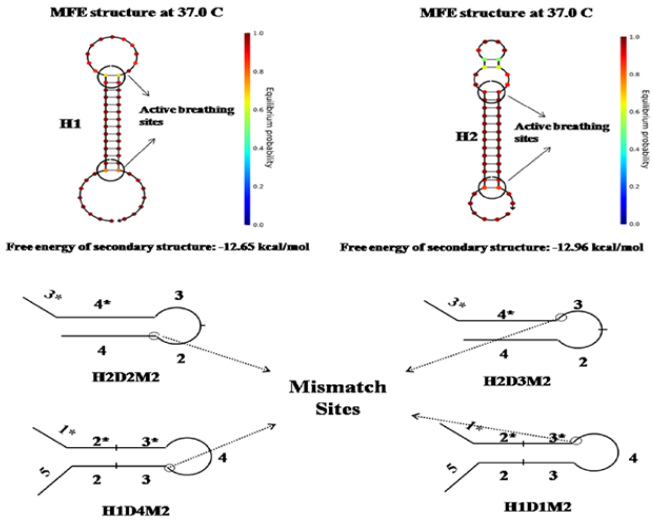
Preparation of Probes. H1 and H2 were designed referring to our recently published work,[S1] based on the principle of the enzyme-free strand-displacement systems. All hairpin probes were heated to 95 °C for 5 min, followed by gradually cooling down to room temperature.[S2,S3] Then the obtained DNA solutions were stored at 4 °C for further use.

Preparation of electrochemical biosensor. The bare gold electrode was polished with 0.05 μm alumina slurries and ultrasonically treated in ultrapure water for a few minutes, followed by soaking in piranha solution (H_2SO_4:H_2O_2=3:1) for 10 min to eliminate other substances. Then, the pretreated electrode was rinsed with ultrapure water and allowed to dry at room temperature. 10 μL aliquot of 200 nM thiolated capture probes was dropped onto the pretreated gold electrode surface and incubated overnight at 4 °C. After washing with the buffer, the electrode was treated with 1 mM MCH for 1 h to obtain well-aligned DNA monolayer and occupy the left bare sites. And the electrode was further immersed in salmon sperm DNA and 1% BSA for 30 min to avoid nonspecific adsorption of DNA and enzyme on the electrode surface.

The exonuclease III-assisted recycling amplification (ERA) reaction was carried out by mixing 1 μL H0 (1 μM), 1 μL Exo III (0.2 units) and varying concentrations of target DNA in 10 mM Tris-HCl buffer (pH 7.8, 10 mM $MgCl_2$, 50 mM NaCl) to a final volume of 20 μL. The reaction mixtures were incubated at 37 °C for 105 min, followed by inactivation of Exo III at 80 °C for 10 min and refolding of residual H0 at 37 °C for 30 min. Then, 20 μL of TNaK buffer (pH 7.5, 20 mM Tris, 140 mM NaCl, 5.0 mM KCl) containing 100 nM H1 and 100 nM H2 was added to initiate mismatched target catalyzed hairpin assembly (CHA) amplification. After 30 min of amplification at 37 °C, the capture probes immobilized on the gold electrode could specifically hybridize with the H1-H2 complexes for another 30 min. Following rinsed thoroughly with diethanolamine (DEA) buffer (pH 9.6, 0.1 M DEA, 1 M $MgCl_2$ and 100 mM KCl) containing 0.05% Tween-20, 10 μL of 0.5 μg mL^{-1} ST-AP was dropped onto the electrode surface and incubated at 37 °C for 30 min. Finally the biosensor was washed with DEA buffer containing 0.05% Tween-20 thoroughly and performed differential pulse voltammetry (DPV) detection in DEA buffer containing 1 mg mL^{-1} of α-NP.

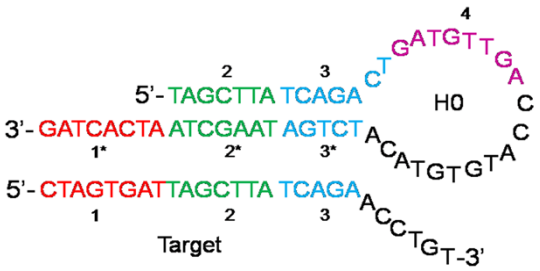
Probe design principle

Although CHA has a lot of advantages, the great background signal is the most trouble problem. The two hairpin substrates in a CHA circuit can potentially react non-specifically even in the absence of target, and this non-specific background degrades the signal-to-noise ratio. The breath sites of hairpin are located at the ends of the hairpin stem. When the hairpin breathed, they indvertently reveal binding sites that can initiate CHA even in the absence of a catalyst strand.[S4] To investigate the effects of the four breath sites on the background signal, two consecutive mismatched base pairs at the end of the stem and the adjacent region were introduced (Scheme S1, sequence design used NUPACK). Mismatched CHA designs can substantially decreased the amounts of uncatalyzed background reactions in CHA amplification reactions.



Scheme S1 The four mismatch positions correspond to the revealed interactions that can initiate CHA reactions between H1 and H2.

The length of 3′-overhang ends of double-stranded DNA has an important effect on Exo III activity [S5,S6]. In this work (Scheme S2), the length of 3′-overhang ends of H0 and target is 8 and 6 bp respectively to resist the cleavage by Exo III. Based on the principle of CHA, the domain 2, 3 and 4 of H0 was specifically designed to initiate CHA reaction. Considering long-chain DNA easily caused secondary structure that could affect the binding of target with the domain 1* of H0, the length of target should not be too long. In conclusion, the length range of target in our work was no less than 13-base. The developed method might be further extended for detection of short DNA (such as BCR-ABL gene) and microRNA in the area of clinical diagnosis and therapy, pathogen detection and environmental monitoring in the future.



Scheme S2 The design principle of target and H0.

Characterization of biosensor fabrication

Electrochemical impedance spectroscopy (EIS) and square wave voltammetric (SWV) measurements were used to characterize the biosensor (Fig. S1A). In the terms of EIS, $[Fe(CN)_6]^{3-/4-}$ was utilized as the redox probe and the semicircle diameter was equal to electron-transfer resistance, Ret. The bare electrode exhibited an almost straight line (curve a), which was characteristic of a diffusion-controlled process. When the capture DNA was self-assembled onto the bare electrode, the Ret increased (curve b) due to the negatively charged phosphate backbone

of the oligonucleotides produced an electrostatic repulsion force to $[Fe(CN)_6]^{3-/4-}$. The Ret obviously increased (curve c) after MCH and BSA were immobilized on the electrode surface, because these biomolecules blocked the electron transfer. Afterwards, when the capture DNA hybridized with the H1-H2 complexes in the absence of the target, the Ret increased slightly (curve d). On the contrary, the Ret increased significantly (curve e) in the presence of target, which indicated that only a few of H1 and H2 could initiate mismatched CHA reaction in the absence of the target, and in the presence of target catalysis, a large amount of H1-H2 complexes were obtained. Also, it proved the successful implement of ERA and mismatched CHA. These results were in a good agreement with those obtained from SWV measurements (Fig. S1B), in which the peak currents varied upon the assembly, hybridization and binding processes. Both results of EIS and SWV proved that the biosensor worked indeed as described in the principle scheme.

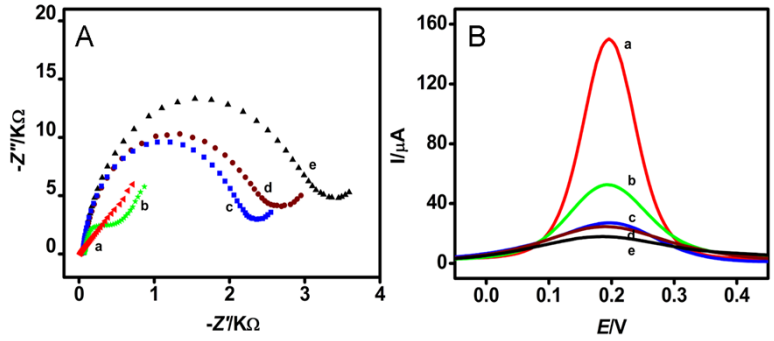


Fig. S1 EIS (A) and SWVs (B) in 0.4 M KCl containing 0.5 mM $Fe(CN)_6^{3-/4-}$ at bare electrode (a), capture DNA modified electrode (b), capture DNA modified electrode after sealed with MCH and BSA (c), after ERA and mismatched CHA without (d) and with target DNA (e), respectively.

Optimization of experimental conditions

For the sake of achieving the excellent assay performance, the different experimental conditions were optimized, and the signal-to-noise ratio was used to evaluate the performance of the biosensor. The amounts of Exo III acting as a primer to initiate an ERA reaction greatly affected the biosensor performance. Therefore, the concentration of Exo III was firstly optimized. The signal-to-noise ratio increased as the increasing concentration of Exo III from 0.001 U to 0.01 U, then decreased from 0.01 U to 0.02 U (Fig. S2A). The reason was that excess concentration of Exo III could digest single-stranded DNA or dsDNA with 3′-protruding termini sometimes. Additionally, the amount of H0 in our work was much lower, which was very sensitive to the concentration of Exo III. Therefore, 0.01 U Exo III was used in all subsequent experiments.

The time of ERA process also played an important role in this experiment. At the Exo III concentration of 0.01 U, the highest signal-to-noise ratio was achieved at 105 min (Fig. S2B). Therefore, 105 min was adopted as the

optimal ERA time. As shown in Fig. S2C, the ratio of H1 to H2 was examined from 4:1 to 1:4. The signal-to-noise ratio reached the maximum at 1:1, so 1:1 was chosen as the appropriate ratio. Fig. S2D depicted the effect of CHA interaction time to the signal-to-noise ratio. As seen, the signal-to-noise ratio increased with the increasing time from 0 min to 30 min, and then decreased from 30 min to 60 min. Hence, the optimized time of CHA interaction was determined to be 30 min for the following works.

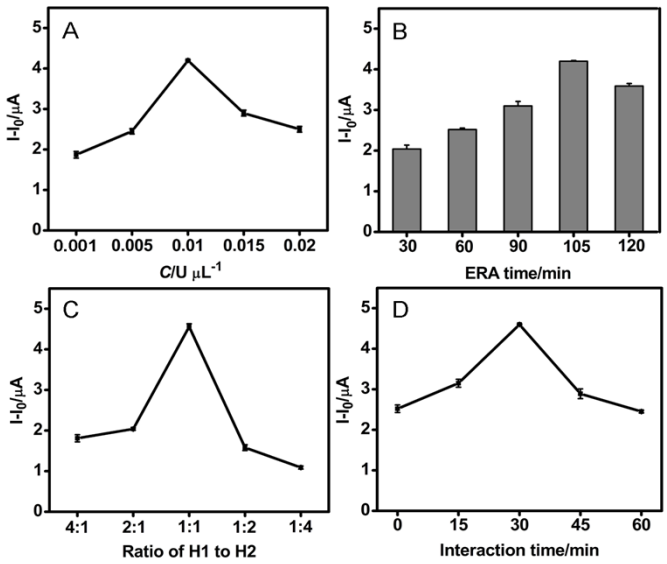


Fig. S2 Dependences of the signal-to-noise ratio on Exo III concentration (A), ERA time (B), Ratio of H1 to H2 (C) and CHA interaction time (D), when one parameter changes while the others are under their optimal conditions.

Supporting references

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Figure captions

Table. S2 Comparison between the proposed assay and other reported methods for DNA detection

Analytical technique	Strategy	Detection limit	Reference
Colorimetry	ERA	2.5 pM	35
Fluorescence	ERA	0.3 pM	36
Colorimetry	CHA	1 nM	37
Fluorescence	CHA	0.2 nM	38
SWV	CHA	20 pM	39
DPV	ERA and CHA	92 fM	This work