Electronic Supplementary Information

Ultrasensitive detection of Nucleic Acids by Template Enhanced Hybridization Followed with Rolling Circle Amplification and Catalytic Hairpin Assembly

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Experimental Section

Reagents. All oligonucleotides used in the present study were synthesized and purified by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China), and the sequences were listed in Table S1. Phi29 DNA polymerase (5 U μL^{-1}, denoted as "polymerase" for short) and the mixture of four dNTPs (2.5 mM for each component) were purchased from TaKaRa Bio Inc (Dalian, China). T4 ligase (5 U mL^{-1}) and the endonuclease (Nb.BsmI, 10000 U mL^{-1}) were purchased from the NEW ENGLAND Biolabs (NEB). TNaK buffer (pH 7.5) contained Tris (20 mM), NaCl (140 mM) and KCl (5 mM). Other chemicals employed were of analytical reagent grade and were used without further purification. Doubly distilled water was used throughout the experiments.

Table S1. DNA sequences used in this work.

Name	Sequences
Target DNA	5'-CCC AAG AGA CCG CGA GAC CT-3'
Assistant DNA	5'-ATC CTC ATA GGT CTC TTG GG-3'
Padlock probe	5-GCT ATG AGG ATC TAT AAA CCC AAC CCG CCG TCC CAA AAC TCG AAT GCC TAG GTC TC-3
Hairpin H1	5'-CTA GGT CTC GCT ATG AGG ATC TAC CAT CGT GTA C TA GAT CCT CAT AGC GAA AGA GCA CCC TTG TCA-3'
Hairpin H2	5'-AGG ATC TAG TAC ACG ATG GTA GAT CCT CAT AGC GAC CAT CGT GTA C-3'
F	5'-TAMRA- TGA CAA GGG TGC TCT TTC GCT ATG -3'
Q	5'-AAG AGC ACC CTT GTC A- DabcyL -3'
Single-base mismatched DNA	5'-CCC AAG ACA CCG CGA GAC CT-3'
Three-base mismatched DNA	5'-CCG AAG AGT CCG CGA GTC CT-3'
Non-complementary DNA	5'-GGG CCT TCG TAA GCC CTG GA-3'
Molecular beacon	5'- TAMRA -GCG CCG TCG GCT ATG AGG ATC TAT AAA CCC CGA CGG CGC- DabcyL -3'

Apparatus. Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out on a Tanon VE-180 cell with the Tanon EPS-300 power supply (Tanon Science & Technology Co., Ltd., Shanghai, China), and the PAGE patterns were imaged on a WD-9413B gel imaging system (Beijing Liuyi

Instrument Factory, Beijing, China). Fluorescence spectra were measured using a Hitachi F−4600 fluorescence spectrometer (Hitachi Ltd., Japan) controlled by FL Solution software. Excitation and emission slits were all set for a 10.0 nm band-pass. The optical path length of a quartz fluorescence cell was 1.0 cm. The fluorophore of 5−carboxytetramethylrhodamine (TAMRA) was excited at 532 nm, and the emission spectra from 550 to 700 nm were collected. The fluorescence intensity at 582 nm was used to evaluate the performance of the proposed assay strategy. All measurements were carried out at room temperature unless otherwise indicated.

Coupling multiple isothermal reactions with amplified target detection and fluorescence detection.

The target DNA determination could be briefly described as follows: the TEHP was performed in 50μL of reaction solution which contained TNaK buffer, 3μL of assistant DNA (1μM), 4μL of linear padlock probe (1μM) and varying concentrations of target DNA. After incubation at 37°C for 30min, 1 μL of 1 × T4 DNA ligase buffer and 0.5 μL of T4 DNA ligase (5.0 U/μL) were added in the DNA solution. This mixture was incubated at 16°C overnight. During this process, the padlock probe was ligated in the presence of target DNA and ligase. Aftereward, the added T4 DNA ligase was denatured by heating at 90°C for 10 min. The resulting product was used for the rolling circle amplification reaction and nicking amplification reaction. 0.5 μL of phi 29 DNA polymerase, 1 μL of dNTPs (10 mM), 0.5μL of Nb.BsmI (5.0 U/μL) and 1.5 μL of 1 × phi 29 DNA polymerase buffer were added to above-prepared mixture. Then the amplification reaction was conducted at 37°C for 4 h, and terminated by heating at 90 °C for 10 min. The product autonomously replicated a multiple machinery cutter cycle and generated accumulated amount of primers. 20 μL of the hairpin H1(50 μM) ,20 μL of the hairpin H2(80 μM) and 20 μL of reporters (FQ) were annealed at 90 °C for 5 min and cooled down to 25 °C at a rate of 0.1 °C/s before use. And the ratio between F and Q was kept to 1:2 to minimize the background fluorescence. After that, H1, H2 and reporters were simultaneously injected into the mixture, and incubated for 2h at 60°C. Finally, the mixture was heated at 90 ℃ for 20 min, then all the enzymes were inactivated. The difference in the fluorescence peak between samples with and without target was used to quantify target molecules in sample.

Gel Electrophoresis.

The samples produced in the process of the RCA reaction were characterized by 15% nondenaturing polyacry-lamide gel electrophoresis (PAGE) analysis (Acr=acrylamide, Bis=N,N'-methylenebisacrylamide; Acr/Bis =29/1). Tris-acetate-EDTA (TAE)(pH=8.5) was used as the separation buffer and the PAGE was carried out at 120 V for 2 h with loading of 9 mL of each sample into the lanes. After Stains-All staining by EB solution for 30 min, gels were photographed by gel image system.

Optimization of Assay Parameters.

Several detection conditions such as the concentrations of Phi29 DNA polymerase, T4 ligase, and nicking enzyme Nb.BsmI; the time and temperature for the CHA, proportion of padlock probe and assistant DNA, were further optimized to improve the detection sensitivity. The final fluorescence was measured in the presence of 10 aM target DNA. To investigate the influence of the concentration of Phi29 DNA polymerase, 0.1 $U\mu L^{-1}$, 0.2 $U\mu L^{-1}$, 0.5 $U\mu L^{-1}$, 1.0 $U\mu L^{-1}$, 1.5 $U\mu L^{-1}$ and 2.0 $U\mu L^{-1}$ were used. As shown in Fig. S1A, as the concentration of Phi29 DNA polymerase increased, the fluorescence intensity increased gradually until 0.5 $U\mu L^{-1}$ was used. The concentration of T4 ligase was another factor which affects the RCA reaction. The dates were shown in Fig. S1B. Though the concentration of T4 ligase was changed from 0.5 $U\mu L^{-1}$ to 2.5 $U\mu L^{-1}$, there was little change in the fluorescence signals. The effect of the concentration of Nb.BsmI on the amplified assay was assessed also. As shown in Fig. S1C, as the concentration of Nb.BsmI increased, the fluorescence intensity increased gradually until 0.50 $U\mu L^{-1}$ was used. The changes of fluorescence intensity with the CHA reaction time were also investigated (Fig. S1D), 90 min was selected as the optimal amplification time for CHA reaction. Several temperatures of reaction (37, 45, 50, 55, 60 and 65℃) were compared to obtain higher detection sensitivity (Fig. S1E). Finally, 60℃ was considered to be the optimal CHA reaction temperature. The proportion of the padlock probe and assistant probe was optimized and the ratio of 4:3 was selected for the assays (Fig. S1F).

Comparison of different methods for DNA detection.

Table S2. The detection limit comparison between our method and other reported ones

Assay	Indicator or amplification	Detection limit	Dynamic range	ref
electrochemical detection	hybridization chain reaction (HCR)	5 aM	5aM-10 pM	1
electrochemical detection	Template enhanced hybridization process (TEHP) and RCA	11 aM	11aM-10pM	2
electrochemical detection	"Y'' junction structure and restriction endonuclease	14 pM	0.1-10 nM	3
surface enhanced Raman scattering	PCR	1 nM	1-5 nM	4
surface enhanced Raman scattering	rolling circle amplification(RCA)	10 pM	5aM-10 pM	5

quartz crystal microbalance	self-assembled DNA nanostructure	0.1 nM	0.5-25 nM	6
surface plasmon resonance imaging	enzyme exonuclease III in conjunction with DNA microarrays	10 pM	10-100 pM	7
surface plasmon resonance imaging	DNA microarray and surface-based RNA transcription	1 fM	1fM- 10pM	8
Scanning Electrochemical Microscopy	DNA Microarrays Enhanced by HRP-Modified SiO_2 Nanoparticles	1pM	1pM- 100nM	9
Chemiluminescent detection	exonuclease III-assisted target recycle- ing amplification and catalytic effect of G-quadruplex- hemin DNAzyme	12 fM	0.02 -1.0pM	10
colorimetric assay	Rolling Circle Amplification Combined with Gold Nanoparticle	70 fM	0.3- 80pM	11
colorimetric detection	gold nanoparticles and hybridization chain reaction amplification	50 pM	0.05 -0.5nM	12
colorimetric DNA assay	Gold nanoparticle-enabled real-time ligation chain reaction	20 aM	50 aM- 5.0 pM	13
Electrochemiluminescence	Dual potential electrochemi-luminescence ratiometic sensor	1.7fM	5.0-1000fM	14
Electrochemiluminescence	Electrochemiluminescence resonance energy transfer system	10 aM	10 aM -10 pM	15
Electrochemiluminescence	Multiwall carbon nanotube with quenching electrochemiluminescence	0.091pM	0.1 pM-1.0 nM	16
fluorescence detection	Molecular beacon-based junction probes	1.6 nM	5pM-100nM	17
fluorescence detection	bimolecular beacon-based enzyme-free cycle	50 pM	0.05 -100nM	18
fluorescence detection	Polymerase chain reaction (PCR)	50 fM	5-200pM	19
fluorescence detection	Magnetic microparticles combined with Exo III	70 pM	0.2-4.0nM	20
fluorescence detection	HCR	1fM	0.2 pM-500nM	21
fluorescence detection	Template enhanced hybridization process (TEHP), RCA and CHA	1.2aM		--

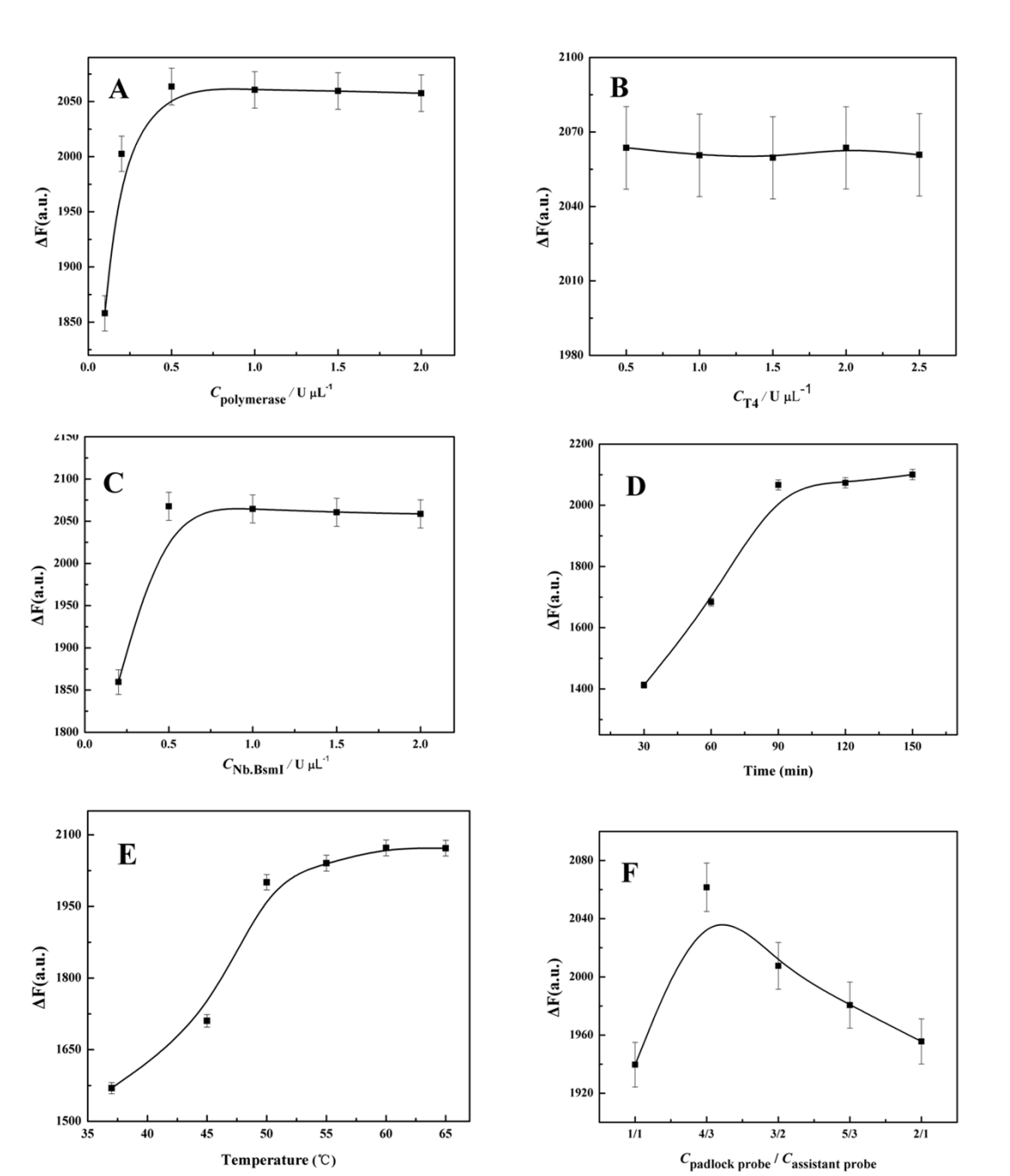
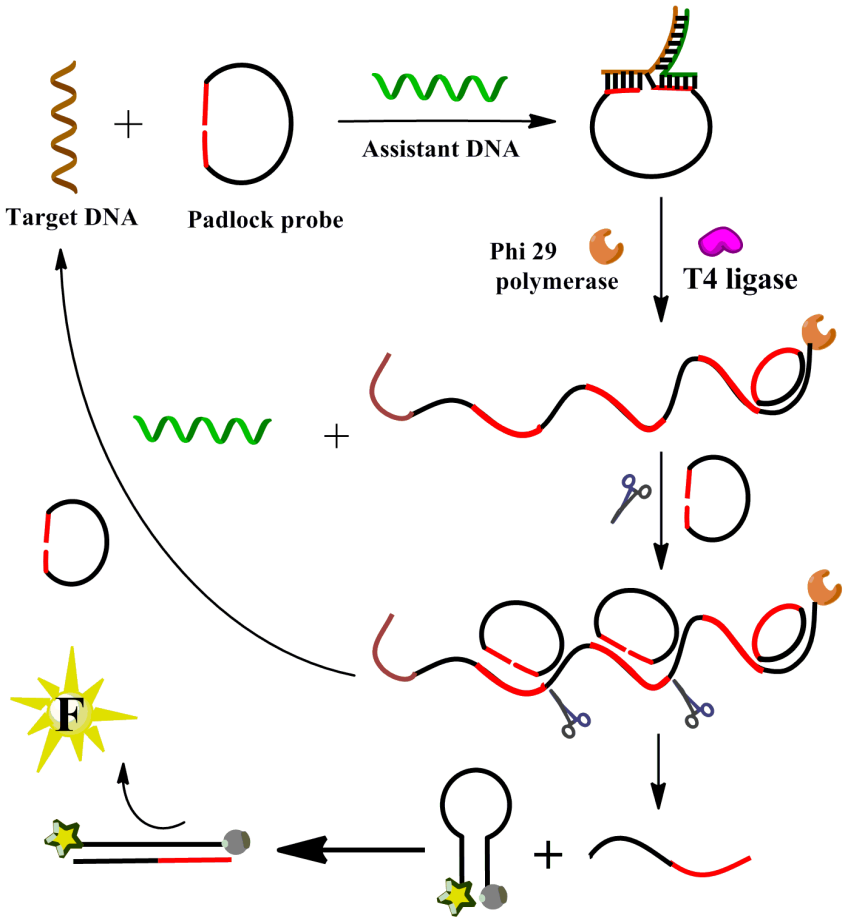


Fig. S1. The relationship between the fluorescence intensity and the concentrations of Phi29 DNA polymerase (A), T4 ligase (B), nicking enzyme Nb.BsmI (C), the time of amplification (D), the temperature (E) and proportion of padlock probe and assistant DNA(F) , $\Delta F=F-F_0$, where F and F_0 are fluorescence intensities of amplification products by the two cycle reaction in the presence and absence of target DNA, respectively. The concentration of DNA was 1.0×10^{-17} M.

The sensitivity of the assay for DNA without the CHA amplification

The sensitivity of the detection for DNA was investigated without the CHA amplification. The experiments were performed in the presence of molecular beacon instead of CHA reaction. As shown in Scheme S1, the TEHP was initiated by the hybridization of the target DNA, assistant DNA, and the padlock probe, forming a stable "Y" junction structure. Upon the T4 ligase introduction, both ends of the base were specifically ligated and the circular template was formed. In this case, the RCA reaction was initiated by adding Phi29 DNA polymerase. Then, extended strand was nicked by the addition of Nb.BsmI, which produced many complementary strands for hybridizing with the molecular beacon. As a result, the molecular beacon was opened and emitted the fluorescent signal. Fluorescence intensity provided a measure for the detection of DNA.



Scheme S1. Schematic diagram for the detection of DNA using molecular beacon as signal probe.

DNA standards at different concentrations were analyzed by the proposed method and the results were

shown in Fig. S2. As the concentration of target DNA increases, large numbers of molecular beacon are opened, resulting in the increase of fluorescence intensity correspondingly. A linear dependence between the intensity and the concentration of DNA was obtained in the range from 10 fM to 1000 fM with a detection limit of 1.0 fM estimated at the 3 times the average standard deviation of the blank response.

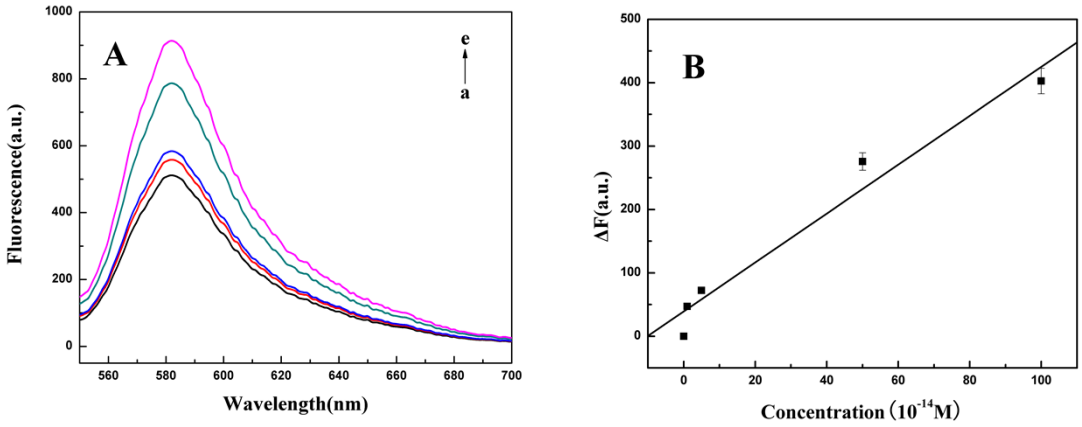


Fig. S2. (A) The fluorescence intensities induced by different concentrations of DNA with the multiple amplified reactions. The curves from a to k contain the concentrations of DNA (10^{-14}M) of (a) 0, (b) 1, (c) 5, (d) 50, (e) 100. (B) Linear relationship between the fluorescence intensities and the DNA concentrations.

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