Electronic Supplementary Information

Homogeneous enzyme-free and entropy-driven isothermal fluorescent assay for nucleic acids based on dual-signal output amplification strategy

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Materials and reagents

All oligonucleotides were supplied by Sangon Biotech Co., Ltd (Shanghai, China) and listed in Table S1. Gel Loading Dye, Purple (6×) was supplied by New England Biolabs Inc. (Beijing, China). Streptavidin-coated microparticles (3 μm) were purchased from Bangs Laboratories (Fishers, IN, USA). 40% acrylamide/bis solution, 19:1 was purchased from Bio-Rad Laboratories Inc. (Shanghai, China). SYBR Gold and 10× Tris-borate-EDTA (TBE) buffer were obtained from Life Technologies (Guangzhou, China). Ethylenediaminetetraacetic acid (EDTA), TEMED, ammonium persulphate (APS), human serum, tris base, urea, $MgCl_2$, KCl, NaCl, and Triton X-100 were obtained from Sigma-Aldrich Inc. (Shanghai, China) and used without further purification. Ultrapure water obtained from a Millipore water purification system (≥ 18 MΩ cm, Milli-Q, Millipore) was used in all assays.

Apparatus

Eppendorf Mastercycler Nexus GX2 (Shanghai, China), ChemiDoc MP Imaging System (Bio-Rad Laboratories Inc., Shanghai, China). Zeiss Axiovert 200M Fluorescent Microscope (LabX, Midland, ON, Canada), SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, Shanghai, China), 96-well Glass Bottom Plate and Nunc 384-well Round Bottom Plate (Yubo Biotech Company, Shanghai, China).

Table S1. Sequences of oligonucleotides used in this work.[a]

Oligonucleotides	Sequences (from 5' to 3')
SS	TGGAGACGTAGGGTATTGAATGAGGGCCGTAAGA GA GCTGTAGATTGGATCG
FS	CGATCCAATCTACAGCTCTCTTACGGCCCTCATTC AATACCCTACG
OS	CCACATACATCATATTCCCTCATTCAATACCCTAC G
AS	CAGTCACTCGATCCAATCTACAGCTCTCTTACGG
T–DNA	CATTCAATACCCTACGTCTCCA
HP1	CACGAGATACTGTTCCCGATCCAATCTACAGC AGATGTGTACCGCTGTAGATTGGATCGAGTGACT G
Biotin–HP1	CACGAGATACTGTTCCCGATCCAATCTACAGC AGATGTGTACCGCTGTAGATTGGATCGAGTGACT G TTTTTTT–biotin
HP2	AGATGTGTACCCGATCCAATCTACAGC GGTACACATCTGCTGTAGA
FR I–FAM	/6-FAM/CCACATACATCATATTCCCT
FR I–Quencher	GTATTGAATGAGGGAATATGATGTATGTGG/IABkF Q/
FR II–FAM	TTGGATCGGGAACAGTATCTCGTGAGC/6-FAM/

FR II–Quencher	/IABkFQ/GCTCACGAGATACTGTTCCC
Sm–DNA	CATTCAATACCCAACGTCTCCA
Tm–DNA	CATTCACTAC*G*CAACGTCTCCA
N–DNA	GAGCTGCTAGATCGACGTCACT

[a]Abbreviation: substrate strand (SS), fuel strand (FS), Output strand (OS), auxiliary strand (AS), target DNA (T–DNA), hairpin 1 (HP1), biotin-hairpin 1 (biotin–HP1), hairpin 2 (HP2), fluorescent reporter (FR), single-base mismatched DNA (Sm–DNA), three-base mismatched DNA (Tm–DNA), and non-complementary DNA (N–DNA).

Real-time fluorescence measurements

(1) Prior to the amplification reaction, the TD probes were first prepared by mixing the three strands (SS, AS, and OS) with equal molar concentration (2.5 μM) in 1× TAE/Mg^{2+} buffer (40 mM Tris-Acetate, 1 mM EDTA, 12.5 mM $MgCl_2$, pH 8.0).

(2) Then the above mixture was heated to 90 ^{o}C for 5 min followed by slowly cooling to room temperature at a rate of 0.1 $^{o}C \cdot s^{-1}$. In addition, 2.5 μM H1, 5 μM H2, 2.5 μM FR I and 2.5 μM FR II were also annealed with the same program.

(3) The amplification reaction was performed in 25 μL of total reaction solution containing 1 μL of 2.5 μM TD, 1 μL of 3 μM FS, 1 μL of 2.5 μM H1, 1 μL of 5 μM H2, 0.5 μL of 2.5 μM FR I, 0.5 μL of 2.5 μM FR II, 1 μL of T−DNA with various concentrations, and 19 μL of 1× TAE/Mg^{2+} buffer.

(4) The reaction mixtures (25 μL) were added into a 384-well plate, which was immediately transferred to a plate reader for fluorescence measurements at 37 ^{o}C for 2 h with the excitation wavelength of 485 nm and emission wavelength of 528 nm.

Preparation method of biotin-HP1/streptavidin-coated microparticles

(1) 50 μL of streptavidin-coated microparticles (3 μm) from stock solution were washed twice with 50 μL binding buffer (20 mM Tris, 1 M NaCl, 1 mM EDTA, 0.0005% Triton X-100, pH 7.5) by centrifuging the microparticles at 10,000 r.p.m. for 3 min, and then decanted the supernatant.

(2) After resuspension of the above microparticles with 50 μL binding buffer, 10 μL of 20 μM biotin-HP1 in TNM buffer (20 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, 0.01% Triton X-100, pH 7.9) was added into the 50 μL microparticles solution.

(3) 60 μL of the above solution containing biotin-HP1 and microparticles was incubated for 15 min at room temperature on a vertical rotator, and then, the sample was washed twice with 50 μL 20 mM TNM buffer by centrifuging the microparticles at 10,000 r.p.m. for 3 min to remove any unbound biotin-HP1 from the biotin-HP1/streptavidin-coated microparticles, followed by resuspension in 50 μL 20 mM TNM buffer and store at 4 ^{o}C (better for freshly use).

CHA reaction on the microparticle surface

To evaluate the feasibility of the biosensing system furtherly, we performed the CHA reaction on the microparticle surface. The amplification reaction was carried out in 50 μL of total reaction solution containing 2 μL of 2.5 μM TD, 2 μL of 3 μM FS, 3 μL of premade biotin-H1/streptavidin-coated microparticles, 2 μL of 5 μM H2, 1 μL of 2.5 μM FR II, 2 μL of 1 μM T−DNA, and 38 μL of 1× TAE/Mg^{2+} buffer. The reaction mixtures (50 μL) were added to a 96-well plate with clear glass bottom, which was immediately transferred to a Zeiss Axiovert 200M Fluorescent Microscope for imaging test at 37 ^{o}C.

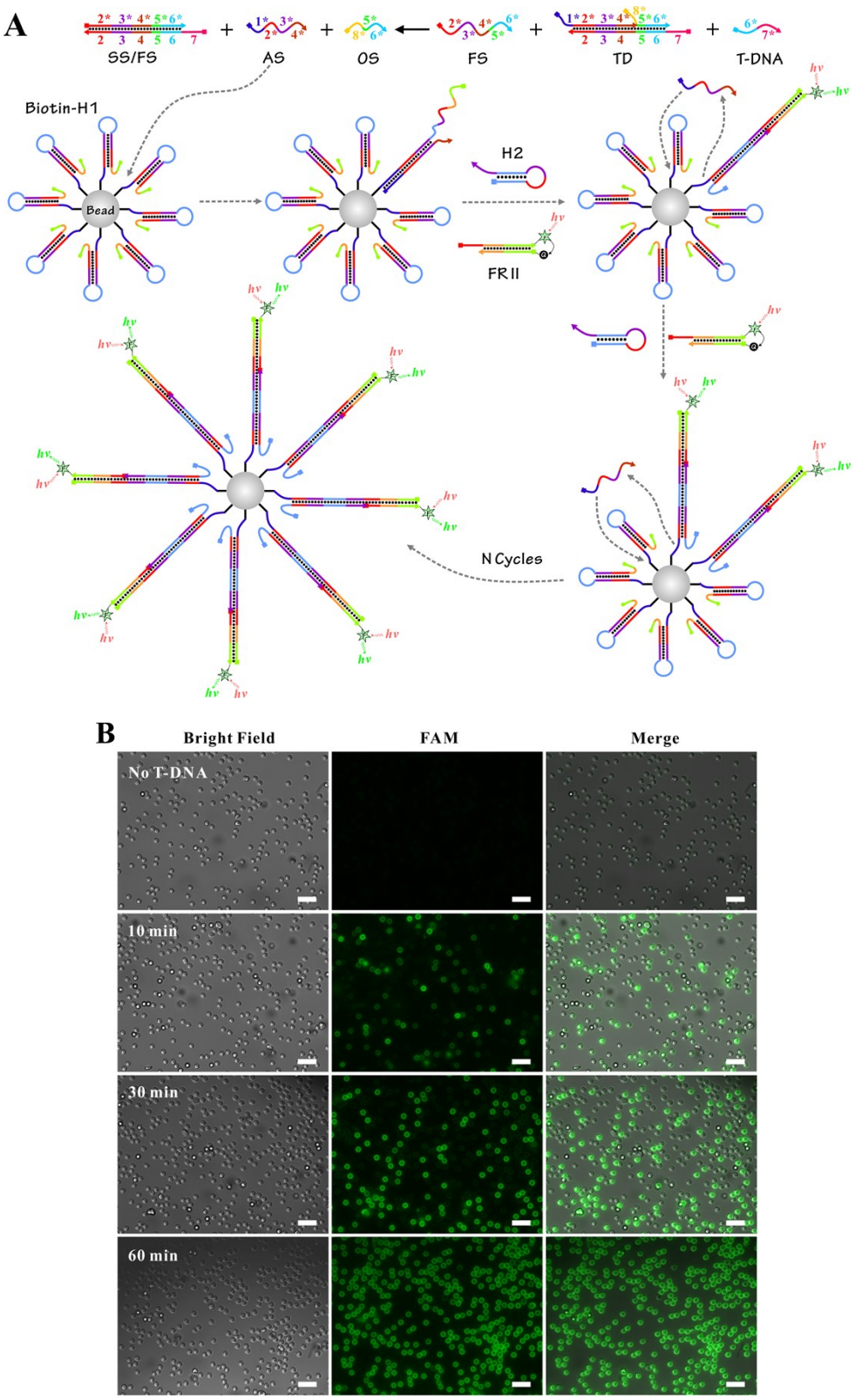

Fig. S1 (A) Schematic illustration of the CHA reaction on the microparticle surface; (B) Images of the biosensing system in the absence of T−DNA and in the presence of 5 nM T−DNA for 10, 30, and 60 min. Scale bar: 10 μm.

Native polyacrylamide gel electrophoresis analysis

Before performing the polyacrylamide gel electrophoresis (PAGE), 10% native PAGE was first prepared using 40% acrylamide and bis-acrylamide solution (19:1) in 1× TBE buffer containing 0.04% APS and 0.1% TEMED and then pre-run the gel at 250 V for 30 min. After the addition of samples in 1× Gel Loading Dye, the gel was operated at 180 V for 2.5 h, followed by staining in SYBR Gold dye solution for 15 min, and then the gel was photographed by the ChemiDoc MP Imaging System.

As shown in Fig. S2, four different sorts of DNA sequence, containing T-DNA (5 nM), single-mismatched DNA (Sm-DNA, 10 nM), three-mismatched DNA (Tm-DNA, 10 nM), and non-complementary DNA (N-DNA, 10 nM) were designed. The N-DNA (curve b) displayed almost the same fluorescence response as the blank solution (curve a). For Sm-DNA, the fluorescence response increased slowly (curve d). Compared with Sm-DNA, the fluorescence response of Tm-DNA increased even more slowly (curve c). After the addition of T-DNA, there was a super-fast increase in the fluorescence response even with a lower concentration (curve e). These results clearly indicated that the proposed fluorescent biosensor possessed excellent specificity for the TDNA assay.

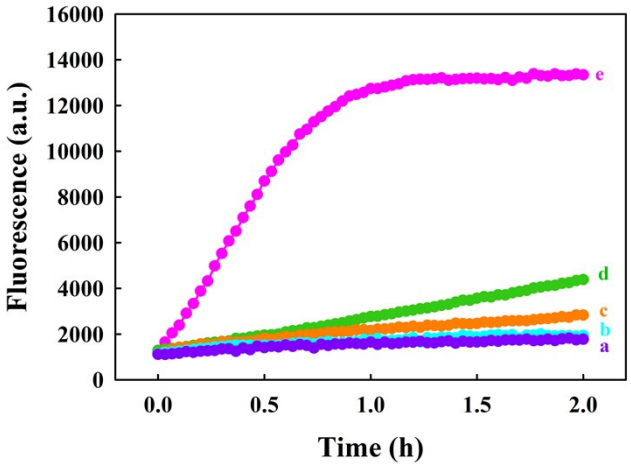

Fig. S2 Specificity of the developed fluorescent biosensor in the blank solution (a), or in the presence of N−DNA (b), Tm−DNA (c), Sm−DNA (d), and T−DNA (e).

Table S2. Comparison of various analytical methods for T−DNA determination.

Analytical Methods	Detection limit	Linear range	Reference
Colormetry	0.57 pM	0.1 nM~10 nM	1
Colormetry	0.6 pM	0.6 pM~10 pM	2
Fluorescence	0.04 nM	0.2 nM~10 nM	3
Fluorescence	3 pM	0.01 nM~10 nM	4
Photoelectrochemistry	0.5 pM	1 pM~100 nM	5
Photoelectrochemistry	94 fM	0.1 nM~8 nM	6
Electrochemiluminescence	0.1 pM	0.1 pM~10 nM	7
Electrochemiluminescence	1 pM	1 pM ~1 nM	8
Electrochemistry	0.3 pM	1 pM~5 nM	9
Electrochemistry	20 pM	20 pM~300 pM	10
Fluorescence	15.6 fM	50 fM~5 nM	this work

Table S3. Comparison of various isothermal nucleic acids amplification techniques.[b]

Amplification techniques	Detection limit	Linear range	Reference
SDA	1.8 pM	2 pM~10 nM	11
SDA	0.1 pM	0.1 pM~10 pM	12
SDA	0.2 pM	0.2 pM~20 pM	13
RCA	28 fM	0.16 pM~1.2 pM	14
RCA	0.5 pM	3.3 pM~27 pM	15
RCA	100 fM	100 fM~1 nM	16
LAMP	0.2 fM	1 fM~1 nM	17
LAMP	0.1 pM	0.1 pM ~100 nM	18
EDC and CHA	15.6 fM	50 fM~5 nM	this work

[b]Abbreviation: strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), entropy-driven catalysis (EDC), catalytic hairpin assembly (CHA).

Table S4. Recovery analysis of T−DNA in 10-fold diluted human serum samples.

Sample (Nos.)	Added (pM)	Found (pM)	Recovery (%)
1	5	4.96 ± 0.21	99.2
2	10	10.13 ± 0.14	101.3
3	50	49.37 ± 0.75	98.74
4	100	102.67 ± 1.89	102.67

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