Supplementary Information

CRISPR/Cas12a platform activated by protospacer adjacent motif-engineered DNA circuit for specific target sensing

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Experimental section

Materials and Reagents

The oligonucleotides were synthesized, purified, and modified by Sangon Biotech Co., Ltd. (Shanghai, China), with their specific sequences detailed in Table S1. The reagents tris (hydroxymethyl) aminomethane (Tris, ≥99.0%), Acry/Bis 40% solution (19:1), and DEPC-treated water were also procured from Sangon Biotech Co., Ltd. (Shanghai, China). Glycerol, ammonium persulfate (APS, ≥98%), and N,N,N',N'-Tetramethylethylenediamine (TEMED, ≥99.0%) were obtained from BBI Co., Ltd. (Shanghai, China). The 20 bp DNA ladder and 6× loading buffer were supplied by TaKaRa Biotech Co., Ltd. (Beijing, China). SYBR™ Gold nucleic acid gel stain (10,000× concentration in DMSO) were procured from Thermo Fisher Scientific Co., Ltd. (Shanghai, China). Laboratory-grade ethylenediaminetetraacetic acid disodium dihydrate ($Na_2EDTA{\cdot}H_2O$, ≥99.0%), boric acid (H_3BO_3, ≥99.5%), magnesium chloride ($MgCl_2$, ≥99.0%), sodium chloride (NaCl, ≥99.0%), potassium chloride (KCl, ≥99.0%), and hydrochloric acid (HCl, 1.2 g/mL at 25°C) were supplied by Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). EnGen LbaCas12a (Cpf1), 10× NEBuffer 2.1, uracil-DNA glycosylase (UDG), human alkyladenine glycosylase (hAAG), human 8-oxoguanine glycosylase 1 (hOGG1), and bovine serum albumin (BSA, 10 mg/mL) were acquired from New England Biolabs (Beijing) Ltd. (Beijing, China). The pesticides acetamiprid (ACE), malathion (MAL), fenpropathrin (FEN), decamethrin (DEC), and imidacloprid (IMI) were supplied by Beijing Putian Tongchuang Biotechnology Co., Ltd. (Beijing, China). The human cervical carcinoma cell line (HeLa) was obtained from the Cell Bank at the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The buffer solutions utilized in the experiments, including Tris-HCl buffer (10 mM Tris, 100 mM NaCl, and 10 mM KCl, pH 7.4), TBE buffer (445 mM Tris, 10 mM EDTA, and 445 mM H_3BO_3, pH 8.3), and TAE/Mg buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, 12.5 mM magnesium acetate, pH 8.0), were prepared using DEPC-treated water.

Table S1 Oligonucleotide sequences used in this study [a]

	Name	Sequence (5' → 3')
Proof of concept	Trigger	TAGC TTAT CAGA CTGA TGTT GA
	H1	TCAA CATC AGTC TGAT AAGC TA TTTA GATC GTTA CGCT AACT ATGA TAGC TTAT CAGA CT
	H1-s1	TCAA CATC AGTC TGAT AAGC TA TTTA CATC GTTA CGCT AACT ATGA TAGC TTAT CAGA CT
	H1-s2	TCAA CATC AGTC TGAT AAGC TA TTTA CTTC GTTA CGCT AACT ATGA TAGC TTAT CAGA CT

	H2	TAAG CTAT CATA GTTA GCGT AACG ATC TAAA TAGC TTAT CAGA CTTA GATC GTTA CGCT AACT ATG
	PAM-lacked H1	TCAA CATC AGTC TGAT AAGC TA GATC GTTA CGCT AACT ATGA TAGC TTAT CAGA CT
	PAM-lacked H2	TAAG CTAT CATA GTTA GCGT AACG ATC TAGC TTAT CAGA CTTA GATC GTTA CGCT AACT ATG
	crRNA	UAAUU UCUAC UAAGU GUAGA UGAUC GUUAC GCUAA CUAUG A
	signal	FAM-TTATT-BHQ1
ctDNA detection	ctDNA	AAAA ATAG GTGA TTTT GGTC TAGC TACA GT
	H1	ACTG TAGC TAGA CCAA AATC ACCT ATTT TT TTTA CATC GTTA CGCT AACT ATGA AAAA ATAG GTGA TTTT GGTC TA
	H2	TATT TTTT CATA GTTA GCGT AACG ATCT AAA AAAA ATAG GTGA TTTT G AGAT CGTT ACGC TAAC TATG
UDG detection	Recognition strand	TTAA AACA UCAG UCUG AUAA GCCC GG
	Trigger	TAGC TTAT CAGA CTGA TGTT GA
	H1	TCAA CATC AGTC TGAT AAGC TA TTTA CATC GTTA CGCT AACT ATGA TAGC TTAT CAGA CT
	H2	TAAG CTAT CATA GTTA GCGT AACG ATC TAAA TAGC TTAT CAGA CTTA GATC GTTA CGCT AACT ATG
ACE detection	Aptamer	TGTA ATTT GTCT GCAG CGGT TCTT GATC GCTG ACAC CATA TTAT GAAG A
	Trigger	GTGT CAGC GATC AAGA ACCG CTGC
	H1	GCAG CGGT TCTT GATC GCTG ACAC TTTA CATC GTTA CGCT AACT ATGA GTGT CAGC GATC AAGA
	H2	CTGA CACT CATA GTTA GCGT AACG ATCT AAAG TGTC AGCG ATCA AGAG ATCG TTAC GCTA ACTA TG

[a] The bases highlighted in yellow represent the sequence of the spacer, while the bases indicated in red denote substitutions. The bases marked in purple correspond to the sequence of the PAM, and the bases depicted in blue illustrate the sequences of the TS and NTS.

DNA circuit-activated CRISPR/Cas12a system

The initial experiment involving the DNA circuit was conducted in 5 μL of Tris-HCl buffer, 5 μL of trigger (0.2 μM), 5 μL of H1 (1.0 μM), and 5 μL of H2 (0.75 μM). This reaction was incubated at 37 °C for 60 minutes. Following this incubation, a Cas12a-mediated cleavage assay

was performed using the reaction products from the DNA circuit, combined with 5 μL of LbaCas12a (100 nM), 5 μL of crRNA (100 nM), 10 μL of signal (1.0 μM), 5 μL of NEBuffer 2.1, and an additional 5 μL of Tris-HCl buffer. This cleavage assay was also conducted at 37 °C for 60 minutes. The DNA circuit was characterized by gel electrophoresis analysis, while the evaluation of the subsequent cleavage assay was performed by recording fluorescence spectra using a fluorescence spectrophotometer (F-7000, Hitachi, Japan). The fluorescence spectra obtained during the experiments were measured within the wavelength range of 505 nm to 650 nm, utilizing an excitation wavelength of 485 nm. The photomultiplier tubes (PMT) were operated at a detector voltage of 400 V, with both the excitation and emission bandwidths configured to 10 nm.

Gel electrophoresis experiments

A 15% nondenaturing polyacrylamide gel solution was initially formulated by combining 10.6 mL of distilled water, 5 mL of 5× TBE buffer, 9.4 mL of a 40% Acry/Bis solution (in a 19:1 ratio), 18 μL of TEMED, and 180 μL of a 0.01% (w/v) APS solution. The resulting gel solution was then transferred into an electrophoresis chamber (JY-CZ-BL, Junyi, Beijing, China) and allowed to polymerize at ambient temperature for approximately five hours. Following polymerization, the reaction samples derived from the DNA circuit experiment were combined with a loading buffer in a 5:1 volume ratio and subsequently loaded onto the gel. Electrophoresis was conducted at a temperature of 15 °C for a duration of two hours, maintaining a constant current of 30 mA. Upon completion of the electrophoresis process, the gel was stained for 40 minutes in a 1× SYBR Gold solution and visualized using a chemiluminescent gel imaging system (FluorChem FC3, ProteinSimple, USA).

CtDNA detection protocol

Initially, a volume of 5 μL of ctDNA at varying concentrations was combined with 5 μL of H1 (1.0 μM) and 5 μL of H2 (0.75 μM) in 5 μL of Tris-HCl buffer to facilitate the execution of the DNA circuit. Subsequently, 5 μL of LbaCas12a (100 nM), 5 μL of crRNA (100 nM), 10 μL of signal (1.0 μM), 5 μL of NEBuffer 2.1, and an additional 5 μL of Tris-HCl buffer were incorporated into the mixture for the cleavage assay. For the one-pot method, the resulting mixture was incubated at 37 °C for 55 minutes, while for the stepwise approach, the two stages were each incubated at 37 °C for 55 minutes.

CtDNA detection in human serum samples

In the context of ctDNA detection in real samples, human blood specimens were procured

from the Shandong Provincial Hospital affiliated with Shandong First Medical University in Jinan, Shandong. The samples underwent an initial centrifugation at a low speed of 600 g for a duration of 5 minutes, followed by a secondary centrifugation at an increased speed of 2000 g for 10 minutes to eliminate any remaining blood cells. Following this processing, 5 μL of the blood sample was sequentially incubated with H1, H2, LbaCas12a, crRNA, signal, NEBuffer 2.1, and Tris-HCl buffer, in accordance with the established protocol.

UDG detection protocol

A recognition probe for UDG was initially developed by combining 5 μL of a recognition strand (1.0 μM) and 5 μL of a trigger (1.0 μM) in 90 μL of TAE/Mg buffer. This mixture underwent an annealing process at 95 °C for 5 minutes, followed by a gradual cooling to 30 °C. Then, 5 μL of the recognition probe, achieving a final concentration of 50 nM, was mixed with UDG at various concentrations to facilitate target recognition and trigger release. The subsequent DNA circuit and cleavage assay was executed in accordance with the previously outlined protocol. In the one-pot method, the resultant mixture was incubated at 37 °C for 75 minutes, while in the stepwise approach, each of the three stages was incubated at 37 °C for 75 minutes.

UDG detection in HeLa cell extracts

In the context of UDG detection in real samples, human HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) that was supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) at a temperature of 37 °C in a 5% CO_2 incubator. After the incubation period, the cells were harvested via trypsinization, followed by washing with phosphate-buffered saline (PBS) at a pH of 7.4, and subsequently centrifuged at 800 rpm for 5 minutes at a temperature of 4 °C. Cellular extracts were prepared using a Nuclear Extract Kit (40010, Active Motif) in accordance with the manufacturer's instructions. The resultant cellular extracts were then analyzed employing the measurement techniques previously outlined.

ACE detection protocol

A recognition probe for ACE was initially formulated at a final concentration of 25 nM. Subsequently, 5 μL of the recognition probe was combined with 5 μL of ACE at varying concentrations to promote target recognition. The DNA circuit was executed in accordance with the previously outlined protocol, and the cleavage assay was performed using 5 μL of LbaCas12a (100 nM), 5 μL of crRNA (100 nM), 10 μL of a signaling molecule (1.0 μM), and 5 μL of NEBuffer 2.1. In the one-pot method, the resulting mixture was incubated at 37 °C for 70 minutes,

whereas in the stepwise approach, each of the three stages was also incubated at 37 °C for 70 minutes.

ACE detection in food samples

In the analysis of real food samples, including tea leaves, milk, spinach, and pork, the samples were first subjected to crushing. Subsequently, they were combined with 2 g of NaCl and 10 mL of acetonitrile. For milk samples, a direct mixture with the aforementioned reagents was performed. For tea leaves, spinach, and pork samples, the resulting mixtures were agitated for three minutes using a vortex mixer, followed by centrifugation at 5000 r/min for five minutes. The supernatant obtained was then transferred to a new centrifuge tube. An additional 5 milliliters of acetonitrile was introduced to repeat the extraction process, and the extracts obtained were subsequently combined. The final food extracts were analyzed using the measurement techniques previously described.

Results and discussion

Feasibility validation of the PAM-engineered DNA circuit-based activation mechanism for CRISPR/Cas12a system

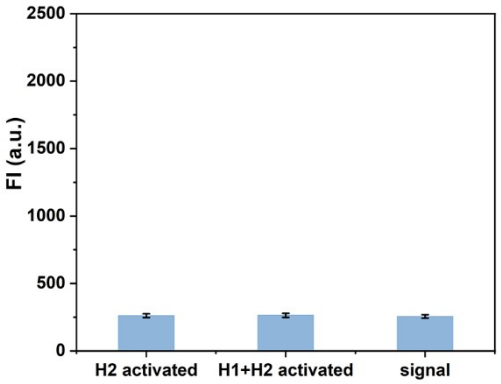

Fig. S1 Fluorescence responses of the signal probe and the mixture comprising LbCas12a, crRNA, H2 or H1+H2, and the signal probe.

The toehold length of H1 is critical for initiating the DNA circuit and was therefore optimized to achieve a higher initiation rate. As illustrated in Fig. S2, toehold lengths of 4, 6, 8, 10, and 12 nucleotides (nt) were evaluated. The fluorescence response increased progressively upon the addition of the trigger sequence; however, an increase in fluorescence was also observed in the blank system at toehold lengths of 10 nt and 12 nt. This phenomenon is attributed to the increased toehold length causing instability in H1, which results in a higher background signal. The signal-to-noise (S/N) ratio reached its maximum at a toehold length of 8 nt. Consequently, an 8-nt toehold was selected as the optimal length for H1. Given that the toehold in H2 serves a similar function, its length was also set to 8 nt.

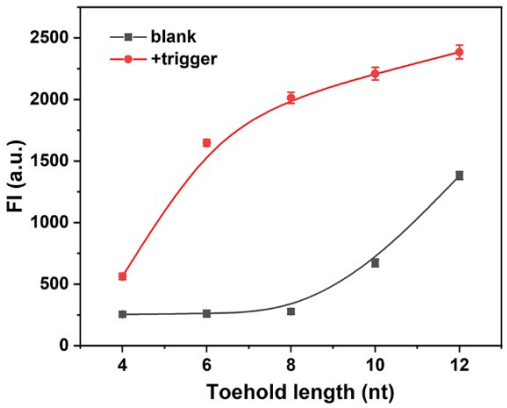

Fig. S2 Fluorescence responses of proposed system at varying toehold lengths of H1.

The loop and stem sizes of hairpins H1 and H2 significantly affect the initiation and progression of the DNA circuit; therefore, these parameters were systematically optimized. Optimization began with the loop and stem sizes of H1. Based on the sequences of the trigger, PAM, and NTS, H1 was designed to contain 60 nt. Excluding the toehold region, the combined length of the loop and stem was set to 58 nt. To prevent the formation of a double-stranded structure between the PAM and NTS, these sequences were incorporated within the loop region. Considering the nucleotide count and design principles, H1 variants with stem sizes of 7, 9, 11, 13, and 15 base pairs were constructed. Due to sequence constraints, the maximum achievable stem length was 15 base pairs. As illustrated in Fig. S3, increasing the stem length of H1 resulted in negligible changes in the fluorescence response of the positive system, whereas the fluorescence signal of the blank system progressively decreased, reaching a plateau at 11 base pairs. This observation is attributed to enhanced stem stability with increasing stem size, which reduces background signal. Consequently, to maximize H1 stability, a stem length of 15 base pairs was selected as the optimal design, corresponding to a loop size of 22 nt. Similarly, the stem size of H2 was optimized. As illustrated in Fig. S4, the S/N ratio attained its maximum values at stem lengths of 18 and 22 base pairs. To enhance the stability of H2, a stem length of 22 base pairs was chosen as the optimal design, corresponding to a loop size of 14 nucleotides.

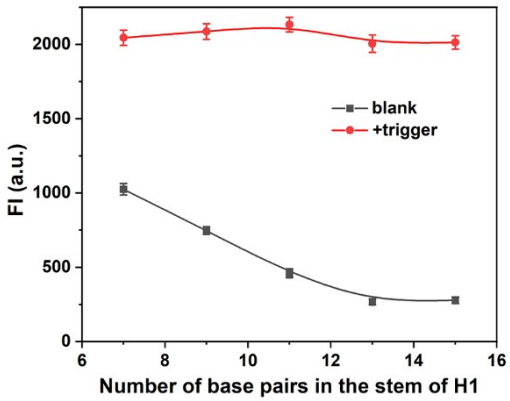

Fig. S3 Fluorescence responses of proposed system at varying stem sizes of H1.

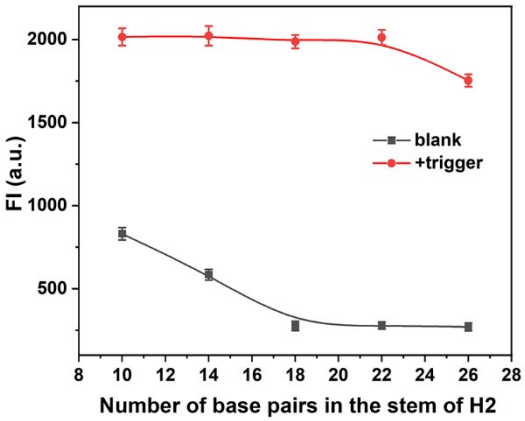

Fig. S4 Fluorescence responses of proposed system at varying stem sizes of H2.

Feasibility and analytical performance for ctDNA detection

Furthermore, an investigation into the optimal reaction time for ctDNA detection was conducted. As depicted in Fig. S5, the fluorescence intensity exhibited a gradual increase with extended reaction time, reaching a plateau at 55 minutes, which was thus designated as the optimal reaction time for ctDNA detection. Additionally, the concentrations of H1 and H2, which are critical for the efficacy of the DNA circuit, were optimized. Figs. S6 and S7 indicate that the optimal concentrations for H1 and H2 were determined to be 1.0 μM and 0.75 μM, respectively. The concentration of CRISPR, which is essential for effective collateral cleavage, was also optimized, resulting in the selection of 100 nM as the optimal concentration, as indicated in Fig. S8. Moreover, the signal concentration was optimized, as shown in Fig. S9; fluorescence responses for both the positive and blank control systems increased progressively from 0.1 μM to 1.0 μM, with the signal-to-noise (S/N) ratio peaking at a signal concentration of 0.5 μM. Consequently, this concentration was chosen as the optimal signal concentration for subsequent experiments.

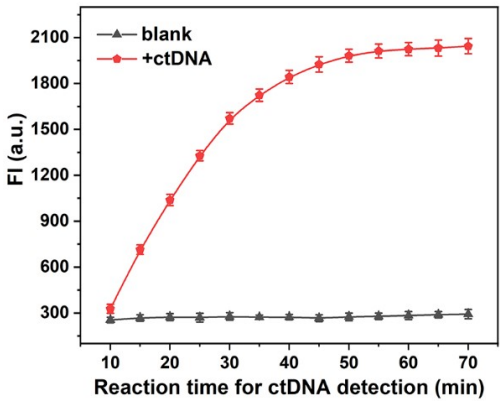

Fig. S5 Fluorescence responses of the proposed approach at different reaction times for ctDNA detection.

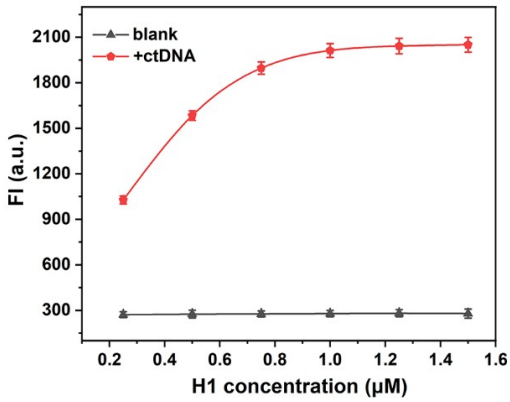

Fig. S6 Fluorescence responses of the proposed approach at different concentrations of H1.

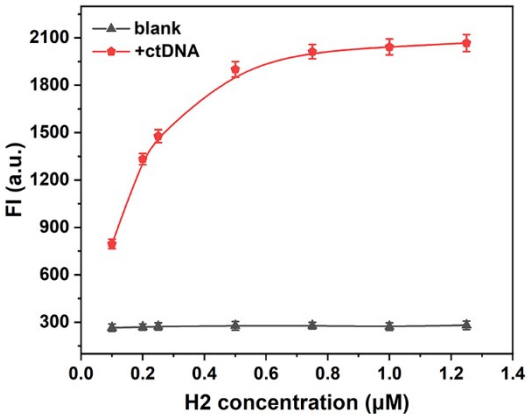

Fig. S7 Fluorescence responses of the proposed approach at different concentrations of H2.

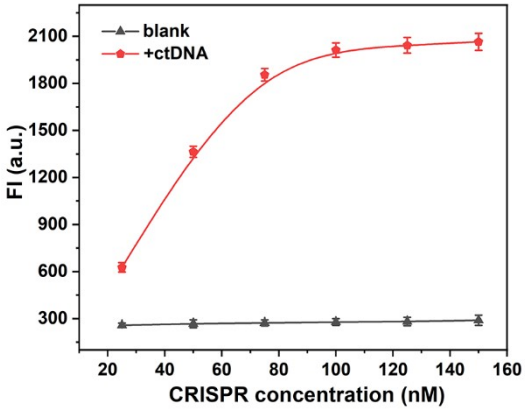

Fig. S8 Fluorescence responses of the proposed approach at different concentrations of CRISPR.

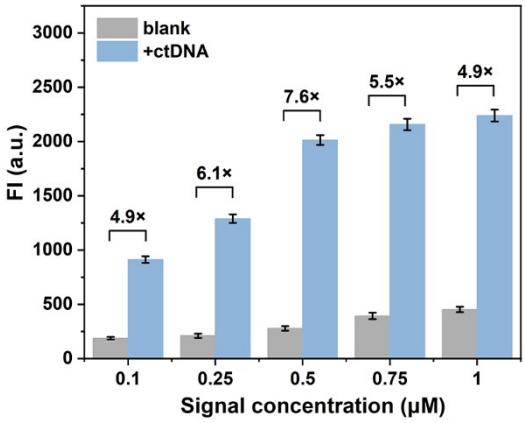

Fig. S9 Fluorescence responses of the proposed approach at different concentrations of signal.

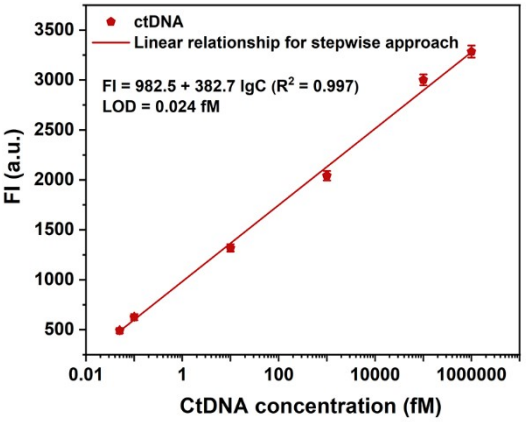

Fig. S10 Linear relationship between fluorescence intensity and ctDNA concentration using the stepwise approach.

Table S2 FI and RSD values for ctDNA detection at different concentrations

Concentration	0.05 fM	0.1 fM	10 fM	1.0 pM	0.1 nM	1.0 nM
FI (a.u.)	478.8	610.2	1319	2042	2917	3220
RSD%	1.20	1.67	3.01	2.25	2.62	3.65

Table S3 Results of the repeatability experiment for ctDNA detection

Concentration	FI (a.u.)				RSD%
	Sample 1	Sample 2	Sample 3	Average	
1.0 pM	2013	1977	2065	2018.3	2.19
10 fM	1302	1334	1287	1307.7	1.84
0.1 fM	601.7	619.3	603.2	608.1	1.60

Table S4 Results of the reproducibility experiment for ctDNA detection

Concentration	FI (a.u.)				RSD%
	Sample 1	Sample 2	Sample 3	Average	
1.0 pM	2013	2057	1962	2010.7	2.36
10 fM	1302	1328	1269	1299.7	2.27
0.1 fM	601.7	605.5	623.6	610.3	1.92

CtDNA assay in clinical blood samples

Table S5 Recoveries of ctDNA in blank serum samples

Concentration	Detected amount				Recovery	RSD%
	Sample 1	Sample 2	Sample 3	Average		
1.0 pM	1.036 pM	1.054 pM	0.982 pM	1.024 pM	102.4%	3.66
10 fM	9.76 fM	9.85 fM	10.21 fM	9.94 fM	99.4%	2.39
0.1 fM	0.1016 fM	0.0983 fM	0.0972 fM	0.0990 fM	99.0%	2.31

Table S6 ctDNA detection in human serum samples

sample	healthy donors (fM)	patients (pM)	healthy donors (fragments 10^3/μL)	patients (fragments10^3/μL)
1	10.46 ± 0.09	1.62 ± 0.07	6.30 ± 0.05	975.24 ± 42.14
2	15.22 ± 0.05	4.25 ± 0.10	9.16 ± 0.03	2558.50 ± 60.20
3	16.71 ± 0.08	3.23 ± 0.12	10.06 ± 0.05	1944.46 ± 72.24
	average		8.51 ± 0.04	1826.07 ± 58.19

Feasibility and analytical performance for UDG detection

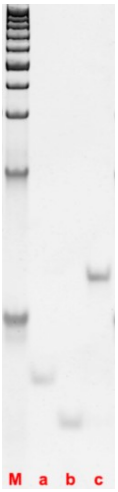

Fig. S11 Gel electrophoresis diagram illustrating the preparation of the recognition probe for UDG. Lane a: recognition strand; lane b: trigger strand; lane c: recognition strand + trigger strand; lane M: the 20bp DNA ladder marker.

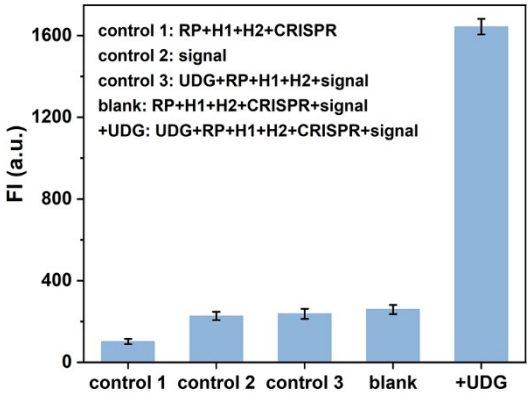

Fig. S12 Fluorescence responses under different experimental conditions for UDG detection.

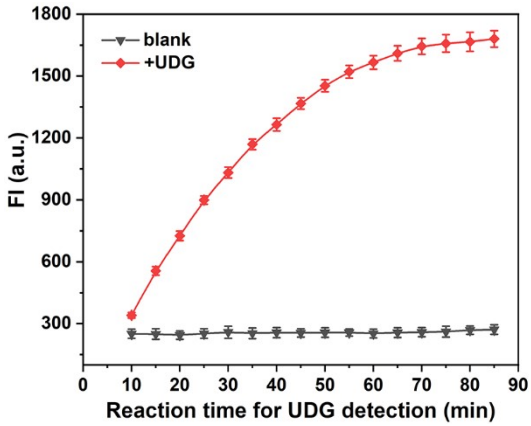

Fig. S13 Fluorescence responses of the proposed approach at different reaction times for UDG detection.

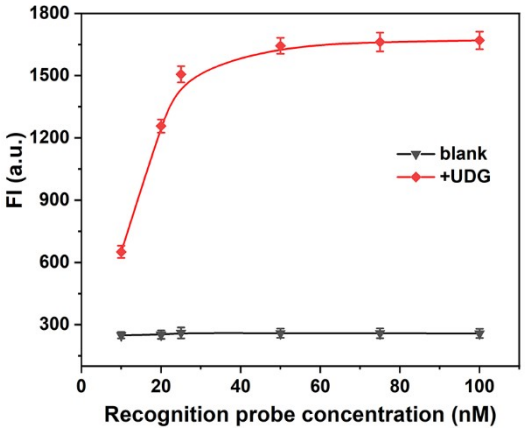

Fig. S14 Fluorescence responses of the proposed approach at different concentrations of recognition probe for UDG detection.

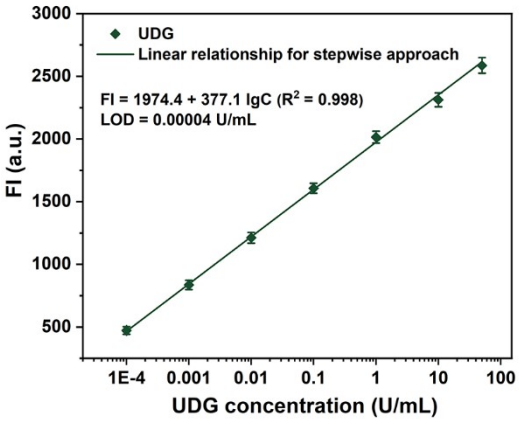

Fig. S15 Linear relationship between fluorescence intensity and UDG concentration using the stepwise approach.

Table S7 FI and RSD values for UDG detection at different concentrations

Concentration (U/mL)	0.0001	0.001	0.01	0.1	1.0	10	50
FI (a.u.)	481.6	829.7	1170	1652	1973	2321	2615
RSD%	1.87	3.01	2.74	2.32	2.20	3.21	3.27

Table S8 Results of the repeatability experiment for UDG detection

Concentration	FI (a.u.)				RSD%
	Sample 1	Sample 2	Sample 3	Average	
1.0 U/mL	1969	1914	2023	1968.7	2.77
0.1 U/mL	1644	1689	1612	1648.3	2.35
0.01 U/mL	1173	1158	1132	1154.3	1.80

Table S9 Results of the reproducibility experiment for UDG detection

Concentration	FI (a.u.)				RSD%
	Sample 1	Sample 2	Sample 3	Average	
1.0 U/mL	1969	2044	1921	1978	3.13
0.1 U/mL	1644	1623	1697	1654.7	2.30
0.01 U/mL	1173	1138	1169	1160	1.65

Table S10 Recoveries of UDG in serum samples

Concentration	Detected amount (U/mL)				Recovery	RSD%
(U/mL)	Sample 1	Sample 2	Sample 3	Average		
1.0	1.029	1.007	0.974	1.003	100.3%	2.76
0.1	0.1013	0.0974	0.0952	0.980	98.0%	3.15
0.01	0.00955	0.00983	0.00989	0.00976	97.6%	1.86

Feasibility and analytical performance for ACE detection

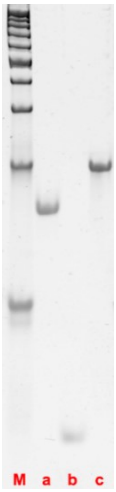

Fig. S16 Gel electrophoresis diagram illustrating the preparation of the recognition probe for ACE. Lane a: aptamer sequence; lane b: trigger strand; lane c: aptamer sequence + trigger strand; lane M: the 20bp DNA ladder marker.

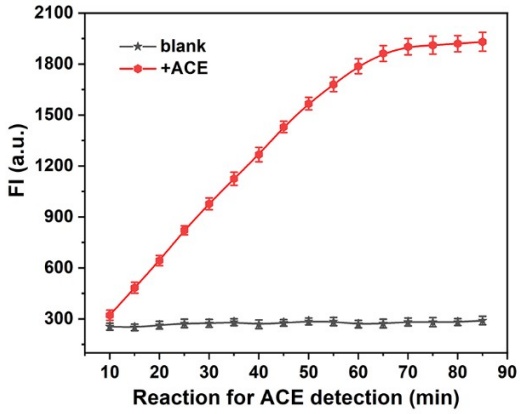

Fig. S17 Fluorescence responses of the proposed approach at different reaction times for ACE detection.

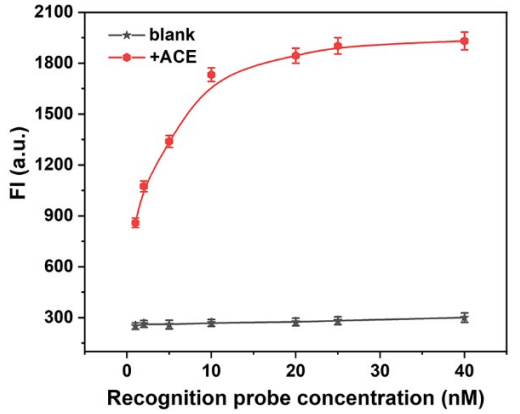

Fig. S18 Fluorescence responses of the proposed approach at different concentrations of recognition probe for ACE detection.

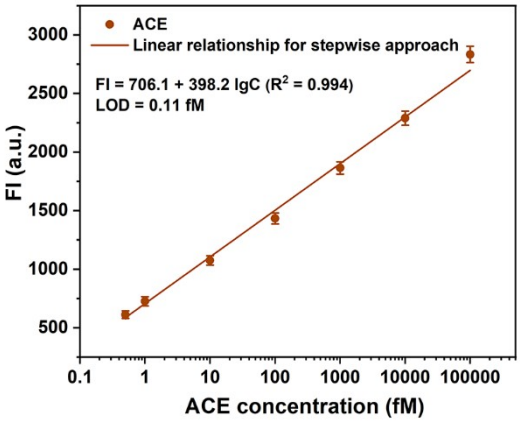

Fig. S19 Linear relationship between fluorescence intensity and ACE concentration using the stepwise approach.

Table S11 FI and RSD values for UDG detection at different concentrations

Concentration	0.5 fM	1.0 fM	10 fM	0.1 pM	1.0 pM	10 pM	0.1 nM
FI	595.8	715.6	1095	1539	1917	2253	2762
RSD	4.08	3.95	2.89	2.64	2.53	2.36	2.16

Table S12 Results of the repeatability experiment for ACE detection

Concentration	FI (a.u.)				RSD%
	Sample 1	Sample 2	Sample 3	Average	
0.1 nM	2750	2711	2679	2733.3	1.76
1.0 pM	1902	1877	1932	1903.7	1.45
10 fM	1084	1123	1076	1094.4	2.30

Table S13 Results of the reproducibility experiment for ACE detection

Concentration	FI (a.u.)				RSD%
	Sample 1	Sample 2	Sample 3	Average	
0.1 nM	2750	2660	2789	2733	2.42
1.0 pM	1902	1834	1952	1896	3.12
10 fM	1084	1061	1133	1092.7	3.36

Table S14 Recoveries of ACE in tap water samples

Concentration	Detected amount				Recovery	RSD%
	Sample 1	Sample 2	Sample 3	Average		
0.1 nM	0.0954 nM	0.0926 nM	0.1019 nM	0.0966 nM	96.6%	4.94
1.0 pM	0.935 pM	0.975 pM	0.986 pM	0.965 pM	96.5%	2.78
10 fM	9.857 fM	9.562 fM	10.04 fM	9.82 fM	98.2%	2.47

ACE assay in food samples

Table S15 Pesticide residue in actual food samples

Food	Sample 1		Sample 2		Sample 3		CNS (mg/kg)
	Amount (mg/kg)	RSD	Amount (mg/kg)	RSD	Amount (mg/kg)	RSD	
Tea leaf	1.8	2.3%	4.5	3.5%	5.7	3.7%	10
Milk	0.012	1.8%	0.0059	2.6%	0.0081	2.0%	0.02
Spinach	1.2	3.3%	1.7	4.1%	0.74	2.9%	1.5
Pork	0.27	2.7%	0.39	3.4%	0.53	1.4%	0.5

[a] CSN: China National Standards (GB 2763-2021)