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Catalytic hairpin assembly-coupled CRISPR/Cas12a biosensor for sensitive detection of melamine in dairy products

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Experimental Section

Reagents and materials. All oligonucleotides used in this study were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The detailed sequences were provided in Table S1. RNA inhibitor, 10×NEBuffer 2.1, SYBR gold nucleic acid gel stain, T7 High Efficiency Transcription Kit, and HiPure RNA Pure Micro Kit were purchased from, MedChemExpress (Shanghai, China), New England Biolabs (Ipswich, MA, USA), Thermo Fisher Scientific Inc. (Waltham, MA, USA), Transgen Biotech (Beijing, China), and Magen Biotech Co., Ltd (Guangzhou, China), respectively. Melamine and cyanic acid were purchased from Aladdin (Shanghai, China). Ammelide and ammeline were purchased from Adamas (Shanghai, China) and Yien Chemtech Co., Ltd. (Shanghai, China). Vitamin B1, Lactose, L-Lys, Ca^{2+}, SO_4^{2-} were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Raw milk, infant formula, and protein powder were acquired from nearby markets.

LbCas12a protein expression and purification. LbCas12a is a subtype of Cas12a that exhibits higher collateral cutting activity than other subtypes.[1] The expression and purification of LbCas12a were conducted in accordance with the established protocol within our laboratory.[2] *Escherichia coli* BL21 (DE3) containing LbCas12a expression plasmid was cultured in 10 mL lysogeny broth (LB) medium supplemented with 1 ‰ kanamycin at 37 ºC and 220 rpm overnight. Subsequently, the culture was transferred to 150 mL LB medium supplemented and incubated for 3～4 hours under identical rotational speed and temperature conditions. Protein expression was induced through the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside, with incubation proceeding at

18 ºC for 12～16 hours, upon attainment of an optical density (OD600) between 0.5 and 0.7. After centrifugation-based cell harvesting, cellular lysis was accomplished via sonication at 4ºC in a buffer comprising 50 mM Tris (pH 8.0), 1.5 M NaCl, 1 mM dithiothreitol (DTT), and 5% glycerol. The protein was isolated using Ni-nitrilotriacetic acid (NTA) agarose chromatography following centrifugal removal of cellular debris. The purified protein was exchanged into a desalting solution (50 mM Tris at pH 8.0, 200 mM NaCl, 1 mM DTT, and 5% glycerol) through ultrafiltration, employing Amicon Ultra-15 (30 K) centrifugal filters. Finally, the purified protein was quantified using the BCA assay kit, diluted to 10 mM, and stored at -80 ºC for subsequent applications.

In vitro transcription and purification of H1 and H2. The DNA templates for **H1** and **H2** transcription were designed with the T7 promoter placed upstream of the **H1** and **H2** sequences. Two synthetic templates were prepared by gradient annealing, starting at 95 ºC for 10 minutes and cooling at 5 ºC per minute to 4 ºC. Then **H1** and **H2** were transcribed and purified by T7 High Efficiency Transcription Kit and HiPure RNA Pure Micro Kit. The obtained **H1** and **H2** were annealed to form hairpin structures, following above annealing procedures.

Gel electrophoresis analysis. SDS-PAGE was used to examine the expression of LbCas12a. Sixteen μL of supernatant, whole protein and purified protein were loaded into 10% SDS-PAGE, running at 120 V constant voltage for 2 h at room temperature.

For non-denaturing PAGE, **T**, HIV-DNA, **H1**, and **H2** were used at 500 nM; **A** or **R** was used at 1 μM; melamine, cyanic acid, ammelide, ammeline were used at 200 μM; Cas12a was used at 1 μM in all experiments. The recognizer **A/T** (or **R/T**) complex was pre-annealed by **A** (or **R**) and **T** at a 2:1 molar ratio to enhance the blocking effect on **T**.

Individual **H1**, **H2** were pre-annealed to form a hairpin structure. The duplex **H1/H2** was pre-annealed with **H1** and **H2,** together.

All mixtures were incubated in 1×NEBuffer 2.1 at 37 °C for 1 h, respectively. Then, 10 μL of each prepared mixture was loaded into 12% non-denaturing polyacrylamide gels and subjected to a 120 V current for 90 minutes. After that, these gels were stained with 2×SYBR Gold nucleic acid for 30 min and then imaged by a Tanon-5200 Chemiluminescent Imaging System.

Fluorescence measurement. For most of fluorescence analysis, the CHA reaction was first carried out in a total volume of 10 μL of reaction solution containing **A/T (**or **R/T)**, **H1**, **H2**, RNase inhibitor and melamine at 37°C for 1 hour. After that, Cas12a-mediated collateral cleavage was initiated by adding a reaction mixture (NEBuffer 2.1, dsDNA activator, LbCas12a, and ssDNA reporter) into the 10 μL reaction solution to total 100 μL mixture. The resulting mixture of 25 nM **A/T (**or **R/T)**, 50 nM **H1**, 50 nM **H2**, 1 U/μL RNase inhibitor, 1×NEBuffer 2.1, 100 nM dsDNA activator, 100 nM LbCas12a, 500 nM ssDNA reporter, and 200 μM melamine (or 100 nM HIV-DNA) was incubated at 37°C for an additional 2 hours. Fluorescence intensity was quantified utilizing a Synergy Mx microplate reader.

For the feasibility and amplification test, the concentrations of **A/T** (or **R/T)**, **H1** and **H2**, were initially set as 100 nM. **A/T** optimal concentrations were 6 nM, 12.5 nM, 25 nM, 50 nM, 100 nM, 200 nM, 400 nM, 600 nM, and 800 nM, respectively. **H1** and **H2** optimal concentrations were 12.5 nM, 25 nM, 50 nM, 100 nM, and 200 nM, respectively.

For sensitivity test, the concentrations of melamine were 0 μM, 0.08 μM, 0.2 μM, 0.4 μM, 0.8 μM, 1 μM, 2 μM, 4 μM, 8 μM, 10 μM, 20 μM, 40 μM, 80 μM, respectively. The

concentrations of HIV-DNA were 0 pM, 0.1 pM, 0.2 pM, 0.5 pM, 1 pM, 2 pM, 5 pM, 10 pM, 20 pM, 50 pM, 100 pM, 1000 pM, 10000 pM, 100000 pM, respectively.

For selectivity test, the concentrations of melamine, cyanic acid, ammelide, ammeline were 200 μM, the concentrations of vitamin B1, lactose, L-Lysine, calcium and sulfate ions were 1.5 μM, 146 mM, 20 mM, 3 mM, 500 mM, respectively; the concentrations of HIV-DNA, HIV-DNA variants, HAV, HBV, HCV, HPV-16, and HPV-18 were 100 nM.

For spiked samples detection, different concentrations of melamine (0.1 and 1 μM) were spiked into common dairy substances, including lactose, vitamin B1, L-lysine, calcium ions, and sulfate ions and their mixtures, respectively. Different concentrations of melamine(0.1, 1 and 10 μM) were also spiked into common products, including raw milk, infant formula, and protein powder, respectively. Different concentrations of HIV-DNA (0.5, 5 and 50 pM) were spiked into human serum. Without pre-treatment, spiked samples were added into CHA system and detected via the same procedure in above florescence measurement.

Statistical analysis. All statistical analyses in this study were performed with GraphPad Prism 8 software. Data are presented as the mean±s.e. of the mean (s.e.m.) from three biological replicates. A two-tailed t test was used to calculate p values, with significance indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and NS, not significant. Recovery rates were calculated based on the equation: Recovery (%) = $(C_n/C_0) \times 100\%$, C_0 represents the concentration of the actual spiked sample, C_n represents the concentration of sample calculated according to the regression equation.

Supplementary Table

Table S1 Oligonucleotide sequences used in this work

Name	*__Sequence (5'-3')__
Melamine trigger (**T1**)	TGAGGTAGTAGGTTGGCACACC
Melamine aptamer1 (**A1**)	GCACACCGATGGCGGTCCTGTTTAGGGGGTGTGCCAA
Melamine aptamer2 (**A2**)	GCACACCGATGGCGGTCCTGTTTAGGGGGTGTGCCAACC
Melamine aptamer3 (**A3**)	GCACACCGATGGCGGTCCTGTTTAGGGGGTGTGCCAACCT A
HIV-DNA	ACTGCTAGAGATTTTCCACAT
HIV trigger (**T2**)	TGAGGTAGTAGGTTGACTGCTA
HIV Recognizer (**R**)	ATGTGGAAAATCTCTAGCAGTCAACC
Melamine **H1**	GGUGUGCCAACCUACUACCUCACAAAAAUUAAAGAAGA AGCACAUGAGGUAGUAGGUU
HIV-DNA **H1**	UAGCAGUCAACCUACUACCUCACAAAAAUUAAAGAAGA AGCACAUGAGGUAGUAGGUU
H2	UACUACCUCAUGUGCUUCUUCUUUAAUUUUUGUGAGGU AGUAGGUU<u>UAAUUUCUACUAAGUGUAGAUCAAAAAUUA AAGAAGAAGCACA</u>
HIV-DNA M1	ACTACTAGAGATTTTCCACAT
HIV-DNA M2	ACTACTAGATATTTTCCACAT
HIV-DNA M3	ACTACTAGATATTTTCGACAT
HAV	GGACTTGATACCTCACCGCC
HBV	TCAGTTTACTAGTGCCATTTGTTCAGTGGT
HCV	ATCTCCAGGCATTGAGCGGGTTTATCCAGGA
HPV-16	AATATGTCATTATGTGCTGCCATATCTACTTCAGAAACT
HPV-18	AGGTACAGGAGACTGTGTAGAAGCACATATTGTTAAATT
Activator-TS	ACTTGTGCTTCTTCTTTAATTTTTGTAAAGACCCC
Activator-NTS	GGGGTCTTTAGAAAAATTAAAGAAGAAGCACAAGT
Reporter	FAM-TTATT-BHQ1

*Sequences with the same color are indicated as complementary sequences, and the underlining indicates crRNA.

Table S2 Comparisons between our method and other methods for the detection of melamine

Analytical methods	Pretreatment	Wash steps	Linear range (μM)	LOD (μM)	Reference
HPLC	Yes	Yes	0.04～1585.4	0.87	[3]
LC-MS	Yes	Yes	—	0.71	[4]
ELISA	No	Yes	—	7.9	[5]
CRISPR/Cas12a aptasensor	No	No	0.1～25	0.038	[6]
CRISPR/Cas14a1-Exo III aptasensor	Yes	Yes	0.004～1	0.0032	[7]
Time-Resolved Fluorescence method	Yes	Yes	0.021～1.2	0.008	[8]
Label-free fluorescence aptasensor	Yes	No	0.5～100	0.12	[9]
Evanescent wave fiber sensor	Yes	Yes	0.1～20	0.15	[10]
Microfluidic ET-NB sensor	Yes	No	0.2～40	0.2	[11]
Signal-on fluorescent aptasensor	Yes	No	7.9～79	0.15	[12]
DNA functional phosphorescent quantum dots	No	No	5～6000	1.6	[13]
Glucose meter-based melamine sensor	No	Yes	0～30	0.53	[14]
Interference-based colorimetric (AgNPs)	Yes	No	0.8～119	11.42	[15]
CHA-coupled CRISPR/Cas12a biosensor	No	No	0.08～10	0.0736	This work

Table S3 Recovery rate of melamine in dairy products

Samples	Spiked (μM)	Found (μM)	Recovery (%)	RSD (n=3, %)
Raw milk	0.1	0.0943±0.0059	94.3386	6.2828
	1	1.0637±0.0789	106.3703	7.4136
	10	10.9063±0.8825	109.0625	8.0915
Infant formula	0.1	0.0984±0.0041	98.4221	4.1720
	1	0.9722±0.1419	97.2243	14.5984
	10	10.8273±1.1755	108.2731	10.8571
Protein powder	0.1	0.0897±0.0068	89.6820	7.6274
	1	0.8737±0.0575	87.3702	6.5843
	10	8.8831±0.8796	88.8314	9.9015

Table S4 Recovery rate of melamine in common substances of dairy products

Samples	Spiked (μM)	Found (μM)	Recovery (%)	RSD (n=3, %)
Mixture	0.1	0.0958±0.0014	95.8245	1.4753
	1	0.9613±0.0343	96.1312	3.5672
Vitamin B1	0.1	0.0985±0.0015	98.4758	1.5597
	1	0.9978±0.0476	99.7839	4.7737
Lactose	0.1	0.0919±0.0035	91.8693	3.7588
	1	0.9314±0.0573	93.1379	6.1513
L-Lys	0.1	0.1012±0.0062	101.1776	6.1752
	1	0.9059±0.1008	90.5942	11.1257
Ca^{2+}	0.1	0.1004±0.0051	100.3925	5.0689
	1	0.9696±0.1017	96.9581	10.4929
$SO4^{2-}$	0.1	0.0997±0.0020	99.7213	2.0422
	1	0.9137±0.0866	91.3705	9.4832

Table S5 Recovery rate of HIV-DNA in serum

Samples	Spiked (pM)	Found (pM)	Recovery (%)	RSD (n=3, %)
Serum	0.5	0.4956±0.0523	99.1109	10.5477
	5	4.9719±0.6393	99.4379	12.8592
	50	50.1247±1.5751	100.2495	3.1423

Supplementary Figure

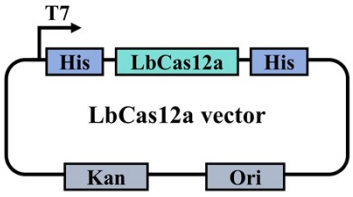

Fig. S1 Schematic of the expression vector for the LbCas12a protein.

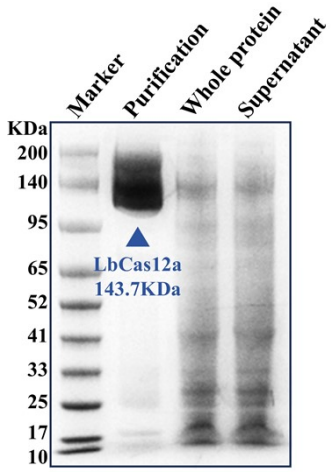

Fig. S2 SDS-PAGE (10%) analysis of purified LbCas12a protein.

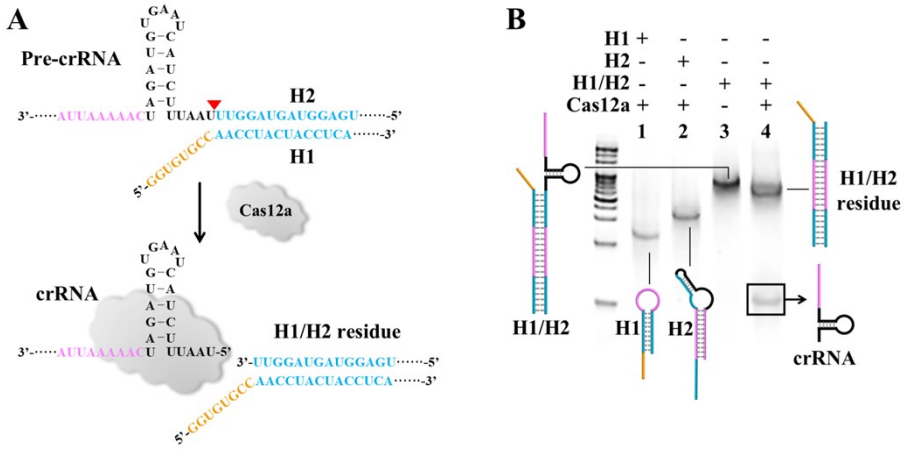

Fig. S3 Schematic illustration (A) and non-denaturing PAGE analysis (B) of pre-crRNA processing activity of Cas12a. Lane M: marker. Lane 1: **H1** + Cas12a. Lane 2: **H2** + Cas12a. Lane 3: **H1/H2**. Lane 4: **H1/H2** + Cas12a.

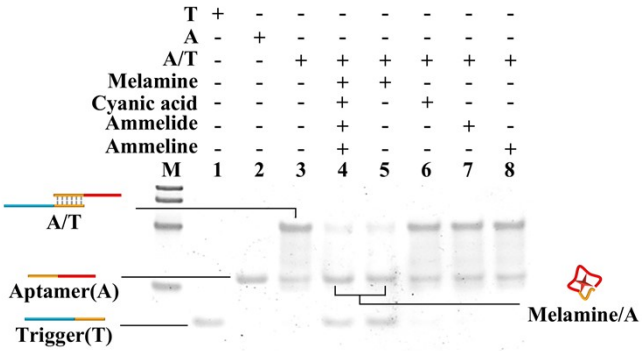

Fig. S4 Non-denaturing PAGE analysis of the specificity of the recognizer **A/T**. Lane M: marker. Lane 1: **T**. Lane 2: **A**. Lane 3: **A/T**. Lane 4: **A/T** + mixture. Lane 5: **A/T** + melamine. Lane 6: **A/T** + Cyanic acid. Lane 7: **A/T** +Ammelide. Lane 8: **A/T** +Ammeline.

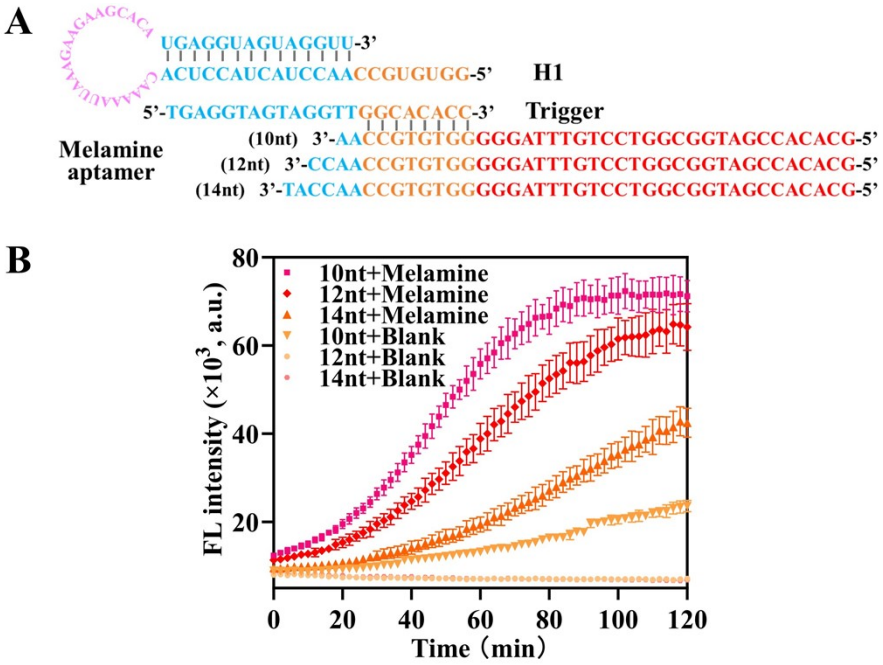

Fig. S5 Influence of complementary lengths on melamine-responsive **T** release. (A) Detailed sequence diagram of **H1**, **T** and **A** with different blocking lengths. (B) Real-time fluorescence curves of different blocking lengths with and without melamine.

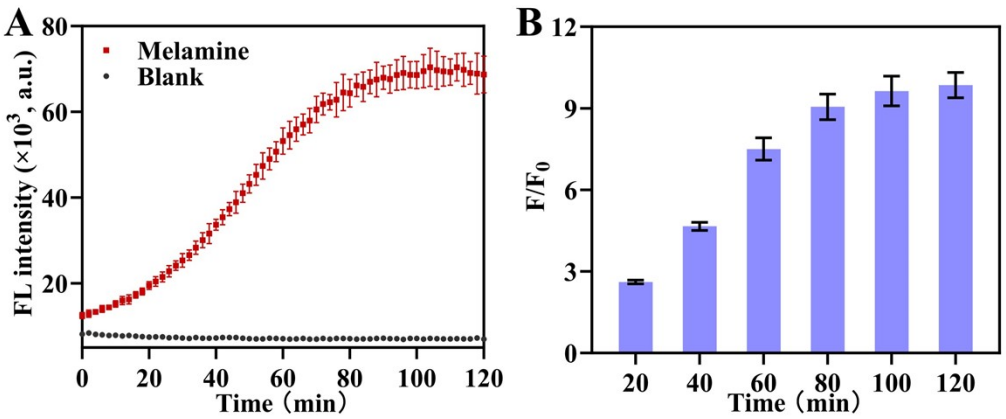

Fig. S6 Optimization of the incubation time with Cas12a. (A) Real-time fluorescence curves of the reaction with and without melamine. (B) Signal-to-noise ratio at different incubation time with Cas12a.

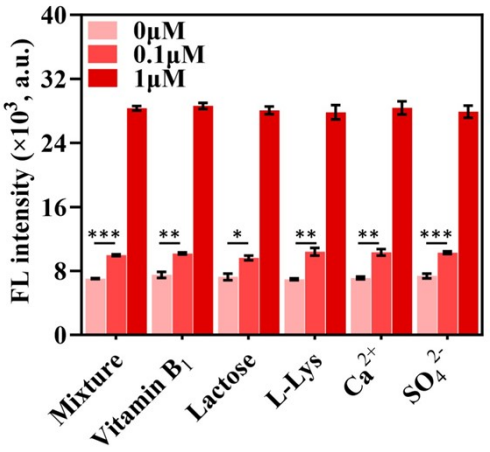

Fig. S7 Fluorescence output of the system in response to different melamine addition (0 μM, 0.1 μM and 1 μM) in the presence of different substances (vitamin B1, lactose, L-Lysine, calcium and sulfate ions, and their mixture).

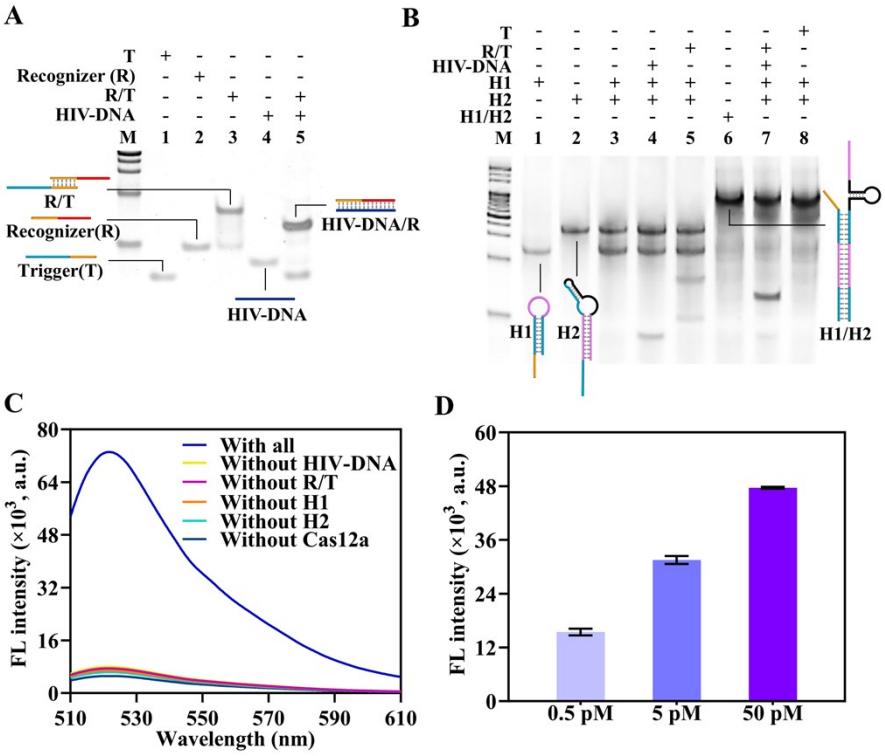

Fig. S8 Feasibility of the biosensor on HIV-related nucleic acid detection. (A) Non-denaturing PAGE analysis of the operation of the recognition module (**R/T**). Lane M: marker. Lane 1: **T**. Lane 2: **R**. Lane 3: **R/T**. Lane 4: HIV-DNA. Lane 5: **R/T** + HIV-DNA. (B) Non-denaturing PAGE analysis of the operation of CHA. Lane 1: **H1**. Lane 2: **H2**. Lane 3: **H1** + **H2**. Lane 4: **H1** + **H2** + HIV-DNA. Lane 5: **H1** + **H2** + **R/T**. Lane 6: **H1/H2**. Lane 7: **H1** + **H2** + **R/T** + HIV-DNA. Lane 8: **H1** + **H2** + **T**. (C) Fluorescence emission spectral curves of the intact system in response to HIV-DNA and the incomplete system without HIV-DNA, **R/T**, **H1**, **H2**, and Cas 12a, respectively. (D) Fluorescence output of the system in response to different spiked serum samples with different HIV-DNA addition, including 0.5 pM, 5 pM and 50 pM.

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