Supplementary Material

A novel CRISPR-Cas12a based fluorescence anisotropy method with high signal-to-background ratio for sensitive biosensing

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1. Experimental Section

1.1 Chemicals and Apparatus

EnGen® Lba Cas12a and 10 × NEB buffer were purchased from New England Biolabs Inc. CRISPR RNA (crRNA) and DNA oligonucleotides were synthesized, purified, and obtained from Sangon Biotech Company, Ltd. (Shanghai, China), and the sequences were listed in Table S1. Diethypyrocarbonate (DEPC), polyacrylate/Bis 40% solution (29:1), 4S Red Plus Nucleic Acid Stain were also ordered from Sangon Biotech Company, Ltd. All the chemicals were analytical grade, and the Milli-Q ultrapure water (18.2 MΩ•cm) was used in the whole study.

Thermomixer C for temperature control were obtained from Eppendorf of Germany. The fluorescence signal was detected by an F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The gel images were collected on BG-gdsAUTO520 under 312 nm UV irradiation. The Atomic force microscopy (AFM) images were recorded by an AFM-Smart SPM (Horiba Jobin Yvon, Inc., France).

1.2 FA analysis

FA was measured by an F-2500 fluorescence spectrophotometer. The excitation wavelength (Ex) was 550 nm, and the emission wavelength (Em) was 585 nm. The slits of excitation and emission were both set at 5 nm, and the voltage was 700 V. *r* was calculated by the following equations:

$$r = \frac{I_{VV} - G \times I_{VH}}{I_{VV} + 2G \times I_{VH}}$$

and

$$G = \frac{I_{HV}}{I_{HH}}$$

where I_{VV} stands for vertical polarization excitation and vertical polarization emission, and I_{VH} is vertical polarization excitation and horizontal polarization emission. The instrumental correction factor *G* is the ratio of the sensitivity of the system to vertical polarization and horizontal polarization, which is related to the emission wavelength.

1.3 Poly acrylamide gel electrophoresis (PAGE)

All DNA nanostructures were analyzed by 12% desaturating PAGE. 10 μL of 1 μM of each DNA stands were mixed with 2 μL of loading buffer. All the prepared gels were run at a constant voltage of 200 V for 30 min in 1 × Tris/borate/EDTA (TBE) buffer, followed by staining with 50 mL of 10% ethidium bromide for 15 min.

1.4 Atomic force microscopy (AFM)

The samples were scanned with Multimode VIII atomic force microscope (Bruker, Inc.) and were imaged using an AFM-Smart SPM (Horiba Jobin Yvon, Inc., France) in tapping mode in fluid. 10 μL of the sample was deposited on the freshly cleaved mica surface. Then AFM images were measured by the center-to-center straight-line distances between the particles in the different structures.

1.5 Synthesis of TDFs

TDFs were synthesized in 1 × TAE buffer (20 mM Tris, 50 mM $MgCl_2$, pH 7.5) by mixing equimolecular of corresponding oligonucleotide strands. The procedures were performed as following: denaturation at 95 °C for 5 min, and then cooled to 25 ℃ for 30 min, finally kept at 25 ℃ for 1h. The final annealed products were stored at 4 °C. The final concentration of TDFs was 1 μM.

1.6 CRISPR-Cas12a reaction

We verified the *trans*-cleavage of CRISPR-Cas12a system through fluorescence spectra and 12% polyacrylamide gel electrophoresis (PAGE). Firstly, we added 20 nM Cas12a, 20 nM crRNA, 25 nM HBV-DNA, 50 nM ssDNA modified carboxyfluorescein (FAM) and black hole quencher (BHQ) simultaneously (FAM-ssDNA-BHQ) and 1× NEB buffer to the centrifuge tube. The mixture was reacted at 37 ℃ for 30 min and then kept at 65 ℃ for 10 min to destroy the activity of protein. Subsequently, the samples were measured by F-2500. The excitation wavelength was set as 490 nm, and emission wavelength was set as 520 nm.

Then, a reaction solution containing 400 nM Cas12a, 400 nM crRNA, 400 nM HBV-DNA, 400 nM H_2 and 1 × NEB buffer was prepared. The mixture was reacted at 37 ℃ for 30 min and then kept at 65 ℃ for 10 min. Next, the trans-cleavage activity of Cas12a was demonstrated by 12% PAGE.

1.7 General procedure

Firstly, H_1 and H_2 were pretreated at 95 ℃ for 5 min, which was cooled to 25 ℃ for 30 min and kept at 25 ℃ for 1h. Finally, it was stored in the refrigerator at 4 ℃ for use. Then, 20 nM Cas12a, 20 nM crRNA, 50 nM H_1, 50 nM H_2, 50 nM initiating DNA (inDNA) and HBV-DNA were mixed together, which was incubated at 37 ℃ for 30 min. Next, the mixture was incubated at 65 ℃ for 10 min to inactivate the activity of Cas12a. After that, 50 nM TDF was injected and controlled

to 200 μL by adding 1 × TAE buffer. The sample was measured after 30 min reaction at room temperature. At last, the *r* was calculated by FA formula.

1.8 Serum sample analysis

Human serum samples were acquired from Southwest University Hospital and strictly abide by the ethics standards of Southwest University Committee (No. yxy2021128). We got informed consents from the blood donor volunteers of this work. We added HBV-DNA into serum to obtain serum solutions with different concentrations of HBV-DNA. Finally, our method was used to detect the above serum solution with HBV-DNA, and the recovery rate was calculated.

2. PAGE analysis and AFM Characterization

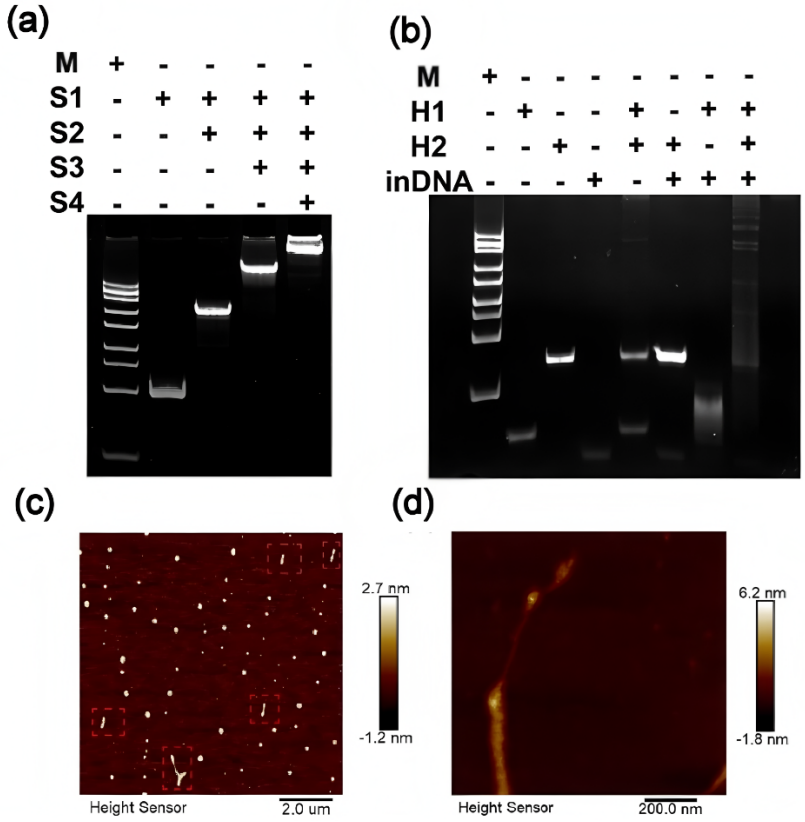

Fig. S1 (a-b) The image of native PAGE (12%) analysis; M is the marker; AFM images of HCR product (c) and DNA nanochain (d).

3. Feasibility analysis

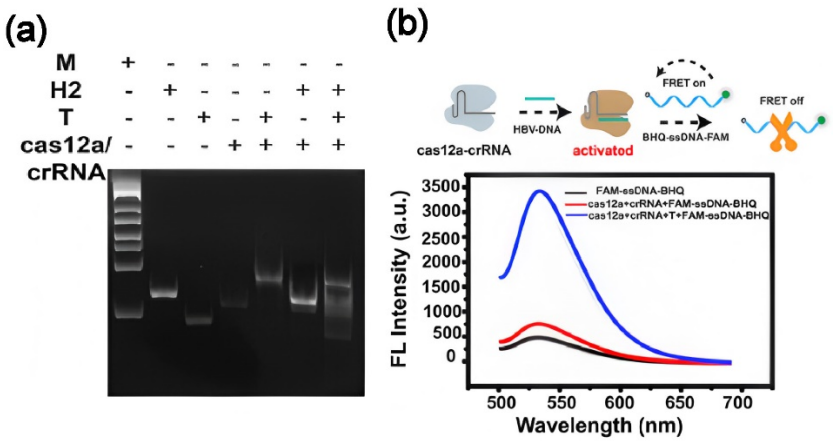

Fig. S2 (a) The image of native PAGE (12%) analysis; M is the marker; T is the target HBV-DNA. (b) The fluorescence measurements of FAM-ssDNA-BHQ in the absence and presence of CRISPR-Cas12a system. λex: 490 nm, λem: 520 nm. Concentrations: HBV-DNA, 25 nM; Cas12a, 20 nM; crRNA, 20 nM; FAM-ssDNA-BHQ, 50 nM.

4. Optimization of the experimental conditions

To reveal the best detection performance of our proposed method, a series of experimental conditions were optimized.

Firstly, we investigated the effect of the concentration of Cas12a-crRNA complex on the FA response. As shown in Fig. S3a, the value of $\Delta r/r_0$ reached to the maximum when the concentration of Cas12a-crRNA was 20 nM. If the concentration of Cas12a-crRNA was too low, TAMRA-H_2 could not be cut completely and the r value was not reduced enough. Nevertheless, if the concentration of Cas12a-crRNA was too high, it could also cleave TAMRA-H_2 without introducing HBV-DNA to activate the activity of Cas12a, resulting in a certain background signal.

Then, the influence of the ratio of Cas12a to crRNA was explored. With the increase of the proportion of crRNA, the $\Delta r/r_0$ showed a trend of first increasing and then decreasing. It indicated that when the radio of crRNA was too high, the inactivated Cas12a would also have a certain cleavage activity, which would eventually cause a high background signal. Therefore, the optimal addition ratio of Cas12a/crRNA was 1:1 (Fig. S3b).

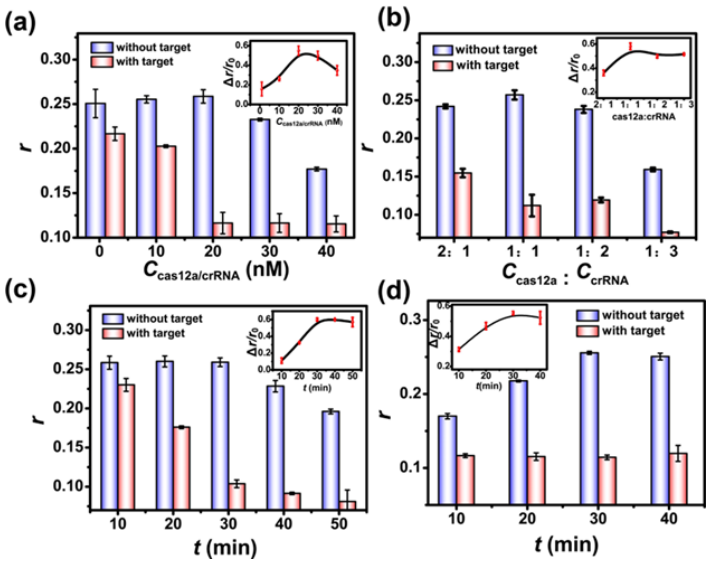

Fig. S3 Optimization of the experimental conditions, including the concentrations of Cas12a-crRNA complex (a), the ratio of Cas12a and crRNA (b), the incubation times of CRISPR-Cas12a system (c), and the incubation times of DNA nanochain (d). Each measurement was triplicated (error bars indicate standard deviation). Concentrations: HBV-DNA, 600 pM; Cas12a, 20 nM; crRNA, 20 nM; H1, 50 nM; H2, 50 nM; inDNA,50 nM; TDF, 50 nM.

Besides, the reaction time was also an important factor because if the reaction time was too short, H_2 would not be cleaved completely, and if the reaction time was too long, even without adding the target CRISPR/Cas12a system, it could be activated to cut H_2. The experimental results (Fig. S3c) showed that when the reaction time of CRISPR-Cas12a system was 30 min, $\Delta r/r_0$ reached its maximum value.

The synthesis of DNA nanochain as an FA amplifier was a key step to enhance the FA signal. Therefore, we explored the formation time of DNA nanochain. As shown in Fig. S3d, when the reaction time was 30 min, the DNA nanochain was fully assembled.

5. Oligonucleotide sequences

Table S1. The list of oligonucleotide sequences

	Sequences (5'to3')
S1	**ATG GAT GAT GTG GTA** ACA TTC CTA AGT CTG AAA CAT TAC AGC TTG CTA CAC GAG AAG AGC CGC CAT AGT A
S2	T ATC ACC AGG CAG TTG ACA GTG TAG CAA GCT GTA ATA GAT GCG AGG GTC CAA TAC
S3	T CAA CTG CCT GGT GAT AAA ACG ACA CTA CGT GGG AAT CTA CTA TGG CGG CTC TTC
S4	T TCA GAC TTA GGA ATG TGC TTC CCA CGT AGT GTC GTT TGT ATT GGA CCC TCG CAT
H1	GTT GCG GCT TCG GGC CCA GAA GCC CGA AGC
H2	GCC CGA AGC CGC AAC GCT TCG GGC TTC TGG-TAMRA
inDNA	GCC CGA AGC CGC AAC ACT
crRNA-HBV-DNA	UAAUUUCUACUAAGUGUAGAU**UACCACAUCAUCCAUAUAAC**
HBV-DNA	TTG GCT TTC AGT TAT ATG GAT GAT GTG GTA

6. Sensors for HBV-DNA assay

Table S2. Comparison of different sensors for HBV-DNA assay

Method	Linear range	LOD	Ref
Fluorescence	0.5-90 nM	5.3 pM	[1]
Colorimetry	50 pM-20 nM	50 pM	[2]
Fluorescence	0.5-50 nM	0.2 nM	[3]
Electrochemistry	10-500 nM	1 nM	[4]
Electrochemistry	0.1 nM-1 μM	0.1 nM	[5]

Fluorescence	4-625 nM	0.97 nM	[6]
Fluorescence	0-500 nM	4 nM	[7]
FA	0.01-1.2 nM	3.8 pM	This work

7. Sensitivities of DNA detection

Table S3. Comparison of the sensitivities of DNA detection by different FA method

Method	Linear range	LOD	Ref
FA	2-8 nM	0.95 nM	[8]
FA	1-20 nM	1 nM	[9]
FA	0.3-12 nM	0.2 nM	[10]
FA	1-800 nM	0.65 nM	[11]
FA	10-50 nM	8 nM	[12]
FA	0.05-2 nM	38.6 pM	[13]
FA	8-40 nM	4.6 nM	[14]
FA	0-25 nM	35 pM	[15]
FA	0.01-1.2 nM	3.8 pM	This work

8. Detection of HBV-DNA in human serum samples

Table S4. Detection of HBV-DNA in human serum samples (n = 3).

Sample	Add (pM)	Found (pM)	Recovery (%)	RSD (%)
Human serum	10	9.8 ± 0.4	91.5 – 102.1	6.2
	100	98.9 ± 7.6	94.4 – 106.5	7.3
	1000	1000 ± 40.4	95.8 – 103.4	3.8

References

1 L. Luo, F. Zhang, C. Chen and C. Cai, *Anal. Chem.*, **2019**, 91, 15748-15756.

2 Y. Tao, Y. H. Lao, K. Yi, Y. Xu, H. Wang, D. Shao, J. Wang and M. Li, *Talanta*, **2021**, 234, 122675.

3 F. Tao, J. Fang, Y. Guo, Y. Tao, X. Han, Y. Hu, J. Wang, L. Li, Y. Jian and G. Xie, *Anal. Biochem.*, **2018**, 554, 16-22.

4 Q. Xiang, J . Y. Huang, H. Y. Huang, W. W. Mao and Z. Z. Ye, *RSC Adv*., **2018**, 8, 1820-1825.

5 H. Zhu, J. Wang and G. Xu, *Cryst. Growth Des.*, **2008**, 9, 633-638.

6 Z. C. Liu, L. Zhang, Y. M. Zhang, R. P. Liang and J. D. Qiu, *Sens. Actuators, B*, **2014**, 205, 219-226.

7 X. Wang, X. Lou, Y. Wang, Q. Guo, Z. Fang, X. Zhong, H. Mao, Q. Jin, L. Wu, H. Zhao and J. Zhao, *Biosens. Bioelectron.*, **2010**, 25, 1934-1940.

8 X. Wang, M. Zou, H. Huang, Y. Ren, L. Li, X. Yang and N. Li, *Biosens. Bioelectron.*, **2013**, 41, 569-575.

9 J. F. Guo, R. M. Fang, C. Z. Huang and Y. F. Li, *RSC Adv.*, **2015**, 5, 46301-46306.

10 J. F. Guo, C. M. Li, X. L. Hu, C. Z. Huang and Y. F. Li, *RSC Adv.*, **2014**, 4, 9379-9382.11 ; J. Chen, Q. Chen, C. Gao, M. Zhang, B. Qin and H. Qiu, *J. Mater. Chem. B*, **2015**, 3, 964-967.

12 Y. X. Liu, X. Xiao, C. H. Li, C. Men, Q. C. Ye, W. Y. Lv, Y. F. Li, C. Z. Huang and S. J. Zhen, *Talanta*, **2020**, 211, 120730.

13 L. Wang, J. Tian, W. Yang, Y. Zhao and S. Zhao, *Luminescence*, **2015**, 31, 573-579.

14 X. Xiao, Y. F. Li, C. Z. Huang and S. J. Zhen, *Chem. Commun.*, **2015**, 51, 16080-16083.

15 J. Zhao, Z. Chu, X. Jin and S. Zhao, *Sens. Actuators B Chem.*, **2015**, 209, 116-121.