Electronic Supplementary Information

Rapid and Sensitive Point-of-care PTS-CRISPR Assay for Food Safety Monitoring of Aflatoxin B1

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Experimental section

Materials and Reagents

High-purity deionized water (18.2 MΩ·cm-1, UPURE, Sichuan) was used throughout this work, but all procedures for the CRISPR-Cas reaction used nuclease-free DEPC water. Tris (2-carboxyethyl) phosphine (TCEP) were obtained from Adamas Reagent, Ltd. (Shanghai, China). LbCas12a (cpf1) and its corresponding 10 × Tolo Buffer 3 were commercially obtained from Tolo Biotech (Shanghai, China). 4-(N-maleimidomethyl) cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt (sulfo-SMCC) was supplied by Bide Pharmatech Ltd. (Shanghai, China). AFB1 were purchased from Benofour Chemical Technology Co., Ltd (Hubei, China). Zearalenone (ZEA), Ochratoxin A (OTA) and Fumonisin B1 (FB1) were purchased from Aladdin Bio-Chem Technology Co. Ltd. (Shanghai, China). The Streptavidin Magnetic Beads (SA-MBs, 1 μm, 10 mg/mL) were bought from MedChemExpress (Shanghai, China). A series of buffers was involved in this work, with details listed as Table S2.

The sequences of the oligonucleotides were shown in Table S1. All HPLC-purified DNA oligonucleotides and crRNAs were supplied by Sangon Biotech Co. Ltd. (Shanghai, China). Human Chorionic Gonadotropin (hCG, L2C00201), was purchased from Linc-Bio Science Co. Ltd. (Shanghai, China). Human chorionic gonadotrophin (hCG) test strips (colloidal gold) were obtained from Wondfo Biotech Co. Ltd (Guangzhou, China).

3K and 10K Amicon Ultra-0.5 NMWL spin filters were purchased from Sigma-Aldrich (Shanghai, China). The UV-vis spectra were recorded by Cytation™ 5 cell imaging multi-mode reader (Bio Tek Instruments Inc., USA). K960 thermal cycler (Heal Force, Inc., Shanghai, China).

Design of "LOCK" DNA structure

In previous studies, most of the AFB1 aptamers used were 50-mer, but Sun and Zhao screened out aptamer with good binding properties and only 29-mer[1, 2]. On this basis, the shorter Aptamer can better maintain the stability of the "LOCK" structure because of the greater degree of hybridization and less possible mismatch. In order to better integrate with the corresponding Activator, appropriate extensions were made on both sides of Aptamer. The sequences of Apt1 and Apt2 are shown in Table S1. Using NUAPCK (nupack.org) for calculation and analysis (Fig), the stem ring structure of Apt1 and Apt2 and the corresponding "LOCK" structure can also be obtained, which verifies the feasibility of the construction of the lock structure on the secondary structure.

Design of crRNA and Activator sequence

The design of crRNA for Cas12a is well established. In general, crRNA consists of two parts: a fixed sequence and a programmable sequence[3]. The fixed sequence constitutes the stem-ring structure for Cas12a to recognize, and the programmable sequence recognizes complementary single - or double-stranded DNA to activate Cas12a's activity. Although there is no specific length requirement for crRNA and its Activator counterpart, the part of recognition hybridization between crRNA and Activator is preferably around 20bp[4]. Therefore, based on the principle of lock structure and aptamer of AFB1, a 26bp Activator was designed (Table S1). Then 20bp of Activator was selected to modify the programmable part of crRNA according to the principle of complementary base pairing (Table S1, underlines part).

hCG-Probe synthesis and purification

hCG was coupled with thiol-modified probe DNA using sulfo-SMCC as the coupling agent[5]. Briefly, 150 μL probe DNA (10 μM) was mixed with 15 μL TCEP solution (10 mM). The mixture was kept at room temperature for 1 h to eliminate the disulfide bonds between the probe DNA. The obtained solution was purified using PBS through Amicon-3K ultrafiltration tube. Meanwhile, 200 μL PBS containing hCG (0.125 mg/mL) was mixed with 20 μL sulfo-SMCC solution (1 mg/mL). The mixture was also incubated at room temperature for 1 h. The obtained solution was purified using PBS through Amicon-10K ultrafiltration tube to remove the unreacted coupling agent. Subsequently, the above two purified solutions were mixed and incubated at 4 ℃ for 36 h. The obtained conjugate was purified using Amicon-10K with NEB 2.1, to remove the excess probe DNA. Then, the conjugate was diluted to the final volume of 200 μL. The prepared hCG-probe solution was stored at 4 ℃ for further use.

Preparation of "LOCK" DNA structure

The AFB1 aptamer and the Cas12a activator were hybridized together as a recognition system, "LOCK" structure (Fig. S1). Apt 1 and Apt 2 are together hybridized with the middle activator strand with calculated melting temperatures of 59.0 and 57.4 °C, respectively, which is thermodynamically stable.

The Apt1, Apt2 and probe DNA were dissolved in the hybridization buffer, the resulting mixture was heated to 90°C for 5 min and then slowly cool to 25 ° C in the thermal cycler to construct the "LOCK" structure. The final concentration of Apt1, Apt2, and probe DNA in the mixed solution were 25, 25, and 12.5 μM, respectively. Apt1 and Apt2 were far in excess of probe DNA to ensure that probe DNA can be fully locked. The resulting Trigger Locking hybrid DNA was stored at 4℃ for further use.

Procedure of PST-CRISPR method for AFB1 detection

Taking 30 μL of the sample in a 200 μL centrifuge tube, add 20μL of the Trigger Locking hybrid DNA (500 nM), 8 μl Cas12a/crRNA complex (containing 0.25 pmol/μL Cas12a, Cas12a: crRNA=1:2), 10 μL hCG probe (all solution were diluted with NEB 2.1), the final system is made up to 80 μL with NEB 2.1. The mixed system was reacted at 37 °C temperature for 25 min and then heated at 65 °C for 5 min to inactivate the enzyme. Finally, the whole reaction system was mixed with an equal volume (80 μL) of 2 × B&W buffer which contained 3 μL of 10 mg mL^{-1} SA-MBs. Upon 25 °C incubation for 30 min, SA-MBs efficiently captured biotin-modified uncleaved hCG-probe and fragments. After magnetic separation, 150 μL of the supernatant was retained for subsequent assays. The pregnancy test strip was immersed in the supernatant and left for 15 s. After leaving the pregnancy test strip for 2 min, the picture of the pregnancy test strip was collected using a smartphone, and image processing and analysis were performed using ImageJ software.

Pretreatment of real samples

Maize flour and peanut oil were obtained from local supermarkets, and processed according to the previous method with some modifications[2]. Briefly, AFB1 was spiked into 1g maize flour at final concentrations of 0.1, 1,10 and 100 ng/g, and 3 mL of extraction solution was added after stirring well. After vigorous shaking for 5 min, the mixture was centrifuged at 12000 rpm for 15 min, and the collected supernatant was filtered with 0.22 μm membrane. Using the same method, 1 mL peanut oil sample containing 0.1, 1, 10, 100 ng/mL AFB1 was extracted. The resulting extraction solution was diluted with the reaction solution for subsequent use to eliminate the influence of methanol.

Table S1. The oligonucleotide sequences used in this study.

Name	Sequence (5'-3')
APT1	*TGC ACG TGT TGT CTC TCT GTG TCT CGT GC*T TGT G
APT2	TGT TG*T GCA CGT GTT GTC TCT CTG TGT CTC GTG C*
Activator	CAC GTG CAC AAC ACA CAA GCA CGA GA
crRNA	UAA UUU CUA CUA AGU GUA GAU UCG UGC UUG UGU GUU GUG CA
T15	SH-C6-TTT TTT TTT TTT TTT-Biotin
T20	SH-C6-TTT TTT TTT TTT TTT TTT TT- Biotin
T25	SH-C6-TTT TTT TTT TTT TTT TTT TTT TTT T- Biotin
T30	SH-C6-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT- Biotin
T35	SH-C6-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TT- Biotin
C30	SH-C6-CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC- Biotin
A30	SH-C6-AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA- Biotin

The italics represent aptamer sequence; the same colors (underlines) represent the complementary sequence.

Table S2. The buffer used in this work.

Buffer	Component
PBS	10 mM PB, 137 mM NaCl and 2.7 mM KCl, pH 7.4
Hybridization buffer	20 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, pH 7.4
10×TOLO buffer	N/A
HEPES	40mM HEPES, 100mM NaCl, 10mM $MgCl_2$, pH 7.4
RNAPol reaction buffer	40 mM Tris-HCl, 6 mM $MgCl_2$, 1 mM DTT, pH 7.6
CutSmart@buffer	50 mM KAc, 20 mM Tris-acetate, 10 mM $MgAc_2$, 100 μg/mL BSA, pH 7.9
NEB 2.1	10 mM Tris-HCl, 50 mM NaCl, 10mM $MgCl_2$, 100 μg/mL BSA, pH 7.9
2×B&W buffer	TE buffer, 2 M NaCl and 0.1% Tween-20

Table S3. Comparison of the proposed PTS-CRISPR method with previous methods for AFB1 detection in this work.

Detection method	**Signal amplification**	**Linear range (pg/mL)**	**LOD (pg/mL)**	**POCT potential**	**Ref**
Electrochemical biosensor	HCR biological amplification	0.01–100	0.00284	Low	[6]
A ratiometric fluorescence	NMOFs-Aptasensor	0–3330	80.0	Average	[7]
Fluorescence anisotropy assay	Streptavidin	18.7–39,034	18.7	Low	[8]
Fluorescence aptsensor	AIE-aptamer-GO system	0–3000	250	Average	[9]
Lateral flow strip	CRISPR-cas	1–100000	2.59	High	This work

Table S4. Recovery results for maize flour and peanut oil samples spiked with different AFB1 concentrations.

Maize Flour (ng/g)	Recovery concentration (ng/g)	Recovery rate (%)	RSD (%)	Peanut oil (ng/mL)	Recovery concentration (ng/mL)	Recovery rate (%)	RSD (%)
0.1	0.085±0.013	84.98	15	0.1	0.090±0.016	90.02	18
1	0.906±0.160	90.61	18	1	0.885±0.028	88.58	3
10	9.691±1.241	96.91	13	10	9.658±2.096	96.58	22
100	98.11±7.809	98.11	8	100	95.64±16.25	95.64	17

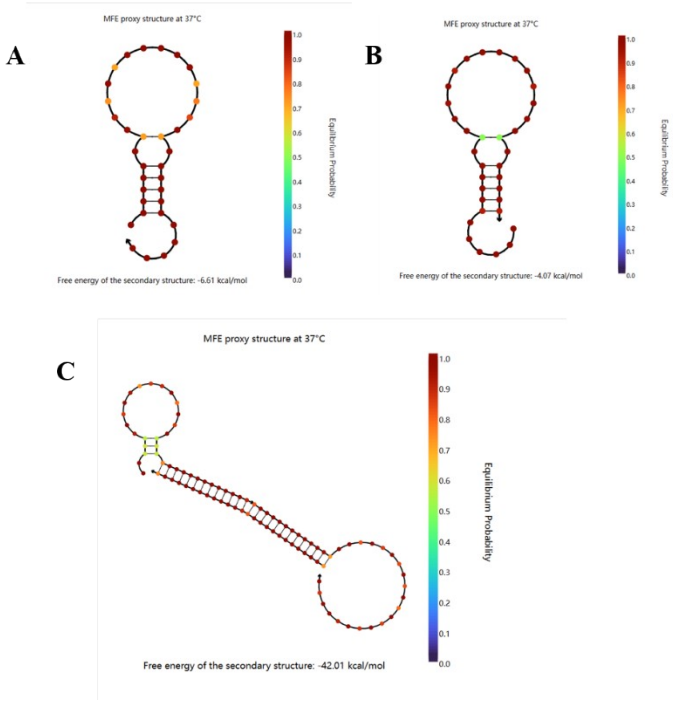

Fig. S1. The secondary structures of (A) Apt1, (B) Apt2 and (C) "Lock" structure.

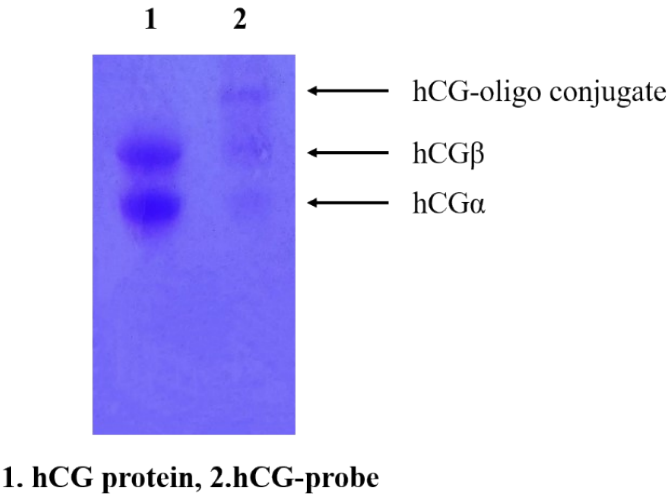

Fig. S2. SDS-PAGE of the conjugation of hCG protein and hCG-probe. The images were taken with a smartphone.

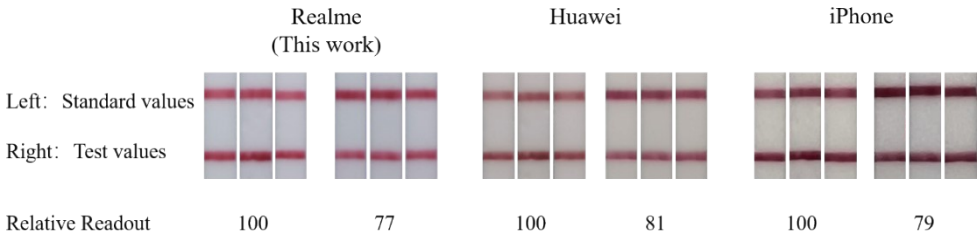

Fig. S3. Comparison of color processing of different mainstream mobile phones.

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