

Programmable Versatile Socket: A Tight-Locking and High-Gain CRISPR/Cas12a Molecular Circuit for Ultrasensitive Sensing of Diverse Targets

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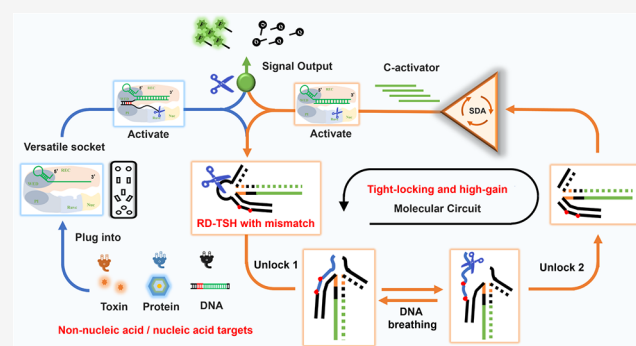
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ABSTRACT: CRISPR/Cas12a has emerged as an important platform for nucleic acid analysis, yet limited catalytic turnover and intrinsic nucleic acid specificity constrain its sensitivity and analyte scope. Herein, a tight-locking and high-gain Cas12a-driven strand displacement amplification (CSDA) molecular circuit is developed as a versatile socket without preamplification for programmable sensing of nucleic acid and non-nucleic acid analytes. CSDA relies on an RNA–DNA three-strand hairpin (RD-TSH) switch containing a 2-nt mismatch. RD-TSH suppresses nonspecific amplification and unintended Cas12a self-activation to ensure tight locking. Screening the number of mismatched bases in RD-TSH and molecular-level mechanistic analyses reveal a DNA breathing-driven two-step unlocking mechanism. Only complete unlocking triggers autocatalytic CSDA, allowing high-gain amplification. The sequence-independent unlocking of RD-TSH confers high orthogonality to CSDA, enabling target-specific modules to be coupled to the CSDA socket as interchangeable plugs via programmable crRNA guides, thus achieving universal detection of both nucleic and non-nucleic analytes. Using *Vibrio parahaemolyticus* DNA, thermostable direct hemolysin, and aflatoxin B₁ as representative targets, CSDA achieved ultrasensitive detection in complex matrices with sensitivity improvements of over 5 orders of magnitude, 42-fold, and 602-fold, respectively. This plug-and-play architecture establishes CSDA as a broadly adaptable and ultrasensitive CRISPR/Cas12a sensing socket, providing a general route toward programmable sensing of diverse analyte classes and a promising strategy for more accurate integrated multitarget analytical platforms.

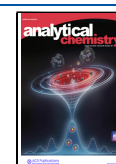


INTRODUCTION

Rapid, sensitive, and facile analytical technologies are of great significance for food-safety prevention and control, thereby motivating the exploration of powerful and versatile molecular sensing platforms.^{1–3} Among the emerging strategies, the CRISPR–Cas12a system has attracted considerable attention in molecular diagnostics owing to its programmable target recognition and efficient trans-cleavage activity.^{4–6} In this system, crRNA-guided recognition of a specific DNA sequence activates nonspecific collateral cleavage, thereby rapidly cutting reporter molecules and amplifying signals, with advantages such as high specificity, strong signal amplification, and operational flexibility. These features make CRISPR–Cas12a highly attractive for the development of simple and efficient sensing technologies. However, CRISPR–Cas12a-based sensing still faces two major challenges in practice, namely insufficient sensitivity and limited universality, owing to the limited catalytic turnover (k_{cat}) and single nucleic acid targeting of Cas12a. In the absence of target preamplification, single CRISPR–Cas systems typically reach only pM-level detection sensitivity.^{7–10} To improve the sensitivity, isothermal ampli-

fication is often coupled with Cas12a, mainly in a two-step process^{11,12} or one-pot formats.¹³ However, two-step workflows are cumbersome and prone to aerosol contamination, whereas current one-pot strategies still exhibit substantial limitations. For example, photocontrolled approaches provide spatiotemporal regulation of amplification and detection,^{14–17} yet they frequently rely on complex chemical modification of crRNA and extensive condition optimization. Likewise, phase-separation-promoting additives such as glycerol or sucrose,^{18,19} or sodium heparin used to disrupt Cas12a–crRNA association and delay Cas12a activation,²⁰ narrow the applicable scope due to additive interference. In parallel, driven by the robust trans-cleavage activity of the CRISPR–Cas12a system, the related sensing technology has been expanded from nucleic acid

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targets to non-nucleic acid analytes, leading to a range of assay formats and applications.^{21–23} For instance, a Y-shaped DNA (Y-COPEs) has been constructed using a PAM topological engineering strategy for allosteric activation of Cas12a, providing a direct and rapid readout of nuclease activity and a programmable universal sensing strategy for non-nucleic acid targets,²² and a steric-hindrance-mediated small-molecule sensing strategy has been developed by truncating and modifying crRNA.²³ These approaches generally modulate Cas12a activity through truncation or conformational reconfiguration of crRNA or dsDNA activators. As a result, different non-nucleic acid targets often require additional sequence and structural design followed by iterative optimization, which complicates the development of a readily transferable universal framework. In addition, universal sensing strategies spanning both nucleic acid and non-nucleic acid targets have been reported,^{24–26} nevertheless, their performance remains limited. In particular, nucleic acid assays remain at pM level, demonstrations largely stay at the proof-of-concept stage, and validation in real samples has not been achieved.

Nucleic acid molecular circuits, as emerging signal transduction and amplification tools, leverage programmable base-pairing and positive-feedback amplification networks to provide a viable pathway for circumventing the low catalytic turnover efficiency of Cas12a and the contamination risks associated with preamplification.^{27,28} However, conventional nucleic acid circuits typically rely on intricate sequence design to couple target recognition with signal output, which not only exacerbates the inherent trade-off between signal leakage and amplification gain,²⁹ but also compromises their orthogonality and transferability across diverse targets.³⁰ Therefore, the key to overcoming the aforementioned bottlenecks lies in constructing a unified molecular architecture that harnesses the amplification advantages of nucleic acid circuits while simultaneously achieving low leakage, high gain, and robust orthogonality, along with plug-and-play adaptability for different target types.

To address the outstanding issues of current CRISPR systems for universal detection that operates without target preamplification and accommodates multiple types of analytes, this work designed a Cas12a-based cascade molecular circuit with an internal positive-feedback loop, which provided strong signal gain, thus offering a new route to overcome the limited sensitivity of conventional Cas12a assays and their reliance on preamplification.^{31,32} The core element of this circuit is a tight-locking RNA–DNA three-strand hairpin (RD-TSH) nucleic acid switch, which was used to construct a self-catalytic molecular circuit termed Cas12a-driven strand displacement amplification (CSDA). By sequestering the 3' end and using RNA to protect the amplification template, RD-TSH suppressed nonspecific amplification, unintended Cas12a self-activation and template damage, thereby establishing tight initial locking. Experimental results and molecular dynamics simulations further revealed that the embedded 2-nt mismatch created a pronounced thermodynamic fluctuation gap between RD-TSH and its unlocked product. This thermodynamic fluctuation gap promoted two-step unlocking of RD-TSH and generated the unlock 2 product, thereby initiating strand displacement amplification (SDA). The amplified products then autocatalytically reactivated Cas12a, closing the positive-feedback loop and enabling triple-cascade amplification with markedly improved sensitivity. Because the Cas12a recognition site responsible for RD-TSH unlocking lay outside the

sequence-specific recognition region (Figure S1a, not the green main trunk), product-driven Cas12a reactivation remained independent of the initial target sequence. This sequence-independent unlocking enabled CSDA to function as a versatile socket: target-specific pretreatment modules converted diverse analytes into sequence-defined nucleotide outputs, which were recognized by programmable crRNAs and coupled to the CSDA sensing module as interchangeable plugs, thereby supporting highly sensitive detection across nucleic acid and non-nucleic acid classes in complex matrices. This substantiated both sensitivity and universality, advancing CRISPR technology toward integrated multiple analysis.

■ EXPERIMENTAL SECTION

Molecular Circuit-CSDA Sensing Analysis with Positive Feedback

The CSDA sensing system comprises two sequential steps: Cas12a-mediated unlocking of RD-TSH (Reaction A) and activation of the positive feedback molecular circuit (Reaction B). Reaction A was initially assembled in a total volume of 5 μ L (for nucleic acid targets) or 10 μ L (for non-nucleic acid targets). The reaction mixture, formulated to a final concentration of 1 $\times$ NEBuffer 2.1, contained 0.8 μ L of 1 μ M LbCas12a, 0.8 μ L of 1 μ M crRNA, 0.25 μ L of 40 U/ μ L RNase inhibitor, 0.4 μ L of 1 μ M RD-TSH, either 1 μ L of nucleic acid targets at varying concentrations or the supernatant from the magnetic separation of aptamer-recognized non-nucleic acid targets. The final volume was adjusted with DEPC-treated water. The mixture was incubated at 42 $^{\circ}$ C for 15 min. Following Reaction A, Component B was added, which included 0.75 μ L of 8 U/ μ L Bst 2.0 DNA polymerase, 0.6 μ L of 10 U/ μ L Nt.BstNBI, 0.4 μ L of 10 mM dNTPs, 4 μ L of 10 μ M FQ-DNA, 0.6 μ L of 100 mM MgSO₄, 0.5 μ L of 10 $\times$ NEBuffer 2.1, 1 μ L of 10 $\times$ isothermal amplification buffer. The final volume was adjusted to 20 μ L with DEPC-treated water, followed by incubation at 42 $^{\circ}$ C for 15 min to initiate the molecular circuit-CSDA with positive feedback (Reaction B).

For 12% polyacrylamide gel electrophoresis (PAGE) analysis, the reaction conditions were identical, except that FQ-DNA signal probe was replaced with a higher molecular weight hairpin DNA to facilitate visualization on the gel.

Polyacrylamide Gel Electrophoresis (PAGE) Analysis

To validate the feasibility of the molecular circuit-CSDA sensing system, the reaction products were analyzed by 12% polyacrylamide gel electrophoresis (PAGE). After a 5 μ L aliquot of the reaction sample was mixed with 1 μ L of 6 $\times$ DNA loading buffer, the mixture was loaded into the wells of a freshly prepared 12% native polyacrylamide gel. Electrophoresis was then performed in 1 $\times$ TBE buffer at a constant voltage of 110 V for 90 min. Finally, the gel was stained with Super Green for 30 min, and the DNA bands were visualized under a blue-light transilluminator (B-BOX, SMOBIO Technology, Inc.).

Molecular Simulation by oxDNA

Construction of Visualization Model. The nucleic acid structures for the locked (RD-TSH) and unlocked (FSNC) states were constructed using oxView. Subsequently, the appropriate force field was selected via the “Force” option, which generated three output files: the configuration file “output.dat”, the topology file “output.top”, and the force field file “output_force.txt”. The nucleotide numbering in the model corresponded to that in the “.top” file, allowing for the identification of specific nucleotide indices through a sequence comparison. Finally, the simulation box dimensions were set to “b = 35 35 35”.

Conformational Relaxation. The constructed model underwent a two-step conformational relaxation using the CPU-based Monte Carlo (MC) and GPU-based Molecular Dynamics (MD) methods. This process eliminated unrealistic geometric features—such as distortions and stretching—introduced during manual structure

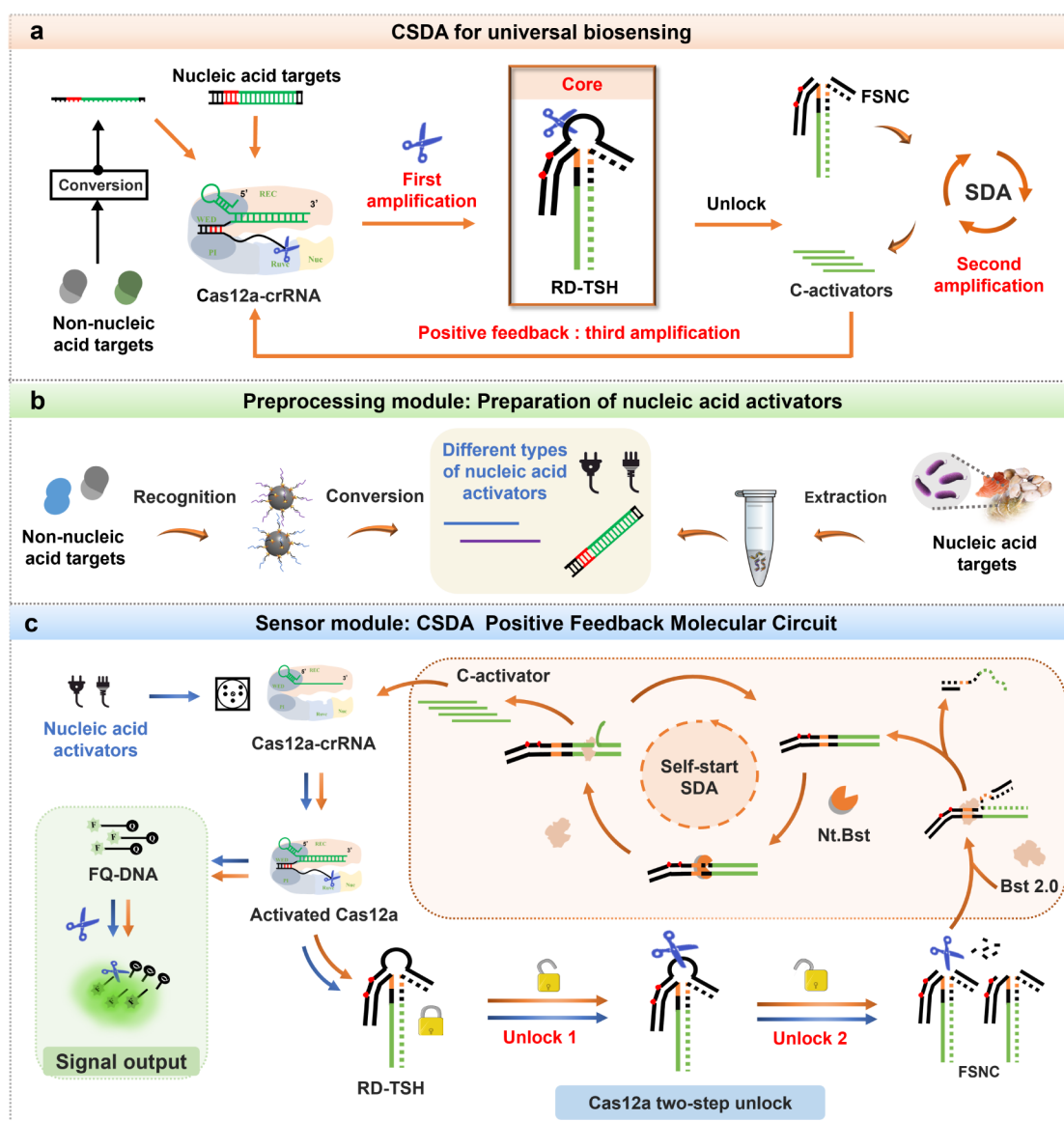


Figure 1. Modular design and operating principle of the self-catalytic CSDA circuit for universal biosensing. (a) Schematic overview of the CSDA-based universal biosensing platform. (b) Target-specific pretreatment strategies for nucleic acid and non-nucleic acid targets. (c) Sensing module mechanism of self-catalytic CSDA circuit with positive feedback, featuring triple cascade amplification (“unlocking–amplification–activation”) and target concentration-dependent fluorescence output.

design, resulting in a stable conformation that was closer to the realistic state. This facilitated the acquisition of correct results in subsequent simulation analyses. The parameters for the MC relaxation were set as “steps = 1e3”, “T = 25 C”, and “simulation_type = MC”. The parameters for the MD relaxation were “steps = 1e6”, “dt = 0.002”, “T = 25 C”, and “simulation_type = MD”.

MD Simulation and Analysis. MD simulations were performed on the fully relaxed structures using oxDNA2 at constant volume and temperature (NVT). The parameters were set as follows: “steps = 1e8”, “dt = 0.002”, “T = 42 C”, and “simulation_type = MD”. The temperature of 42 °C was used to better mimic the experimental conditions for observing conformational changes. A salt concentration of 1 M NaCl was maintained in all systems. The oxDNA2 analysis tools, along with the sequence-dependent parameters file “seq_dep_file = oxDNA2_sequence_dependent_parameters.txt”, were used to calculate the distances D1–D5 between the five thymine (T) bases at the vertex of the loop and the guanine (G) bases at the corner of the main RNA sequence for the three nucleic acid switches (RD-TSH0 to RD-TSH2). The spatial distances of the loops were analyzed to evaluate the successful cleavage probability (SP) and high-

efficiency cleavage probability (EP) of Cas12a for the different switches. The calculation formulas for SP and EP are as follows:

For a successful cleavage event:

$$\text{Input D} \geq 2.49 \text{ nm, Output} = 1$$

$$\text{Input D} < 2.49 \text{ nm, Output} = 0$$

$$\text{SP} = \frac{n(1)}{n(1) + n(0)} \quad (1)$$

For an efficient cleavage event:

$$\text{Input D} \geq 2.94 \text{ nm, Output} = 1$$

$$\text{Input D} < 2.94 \text{ nm, Output} = 0$$

$$\text{EP} = \frac{n(1)}{n(1) + n(0)} \quad (2)$$

Here, $n(1)$ represents the number of output events with “output = 1”, and $n(0)$ represents the number of output events with “output =

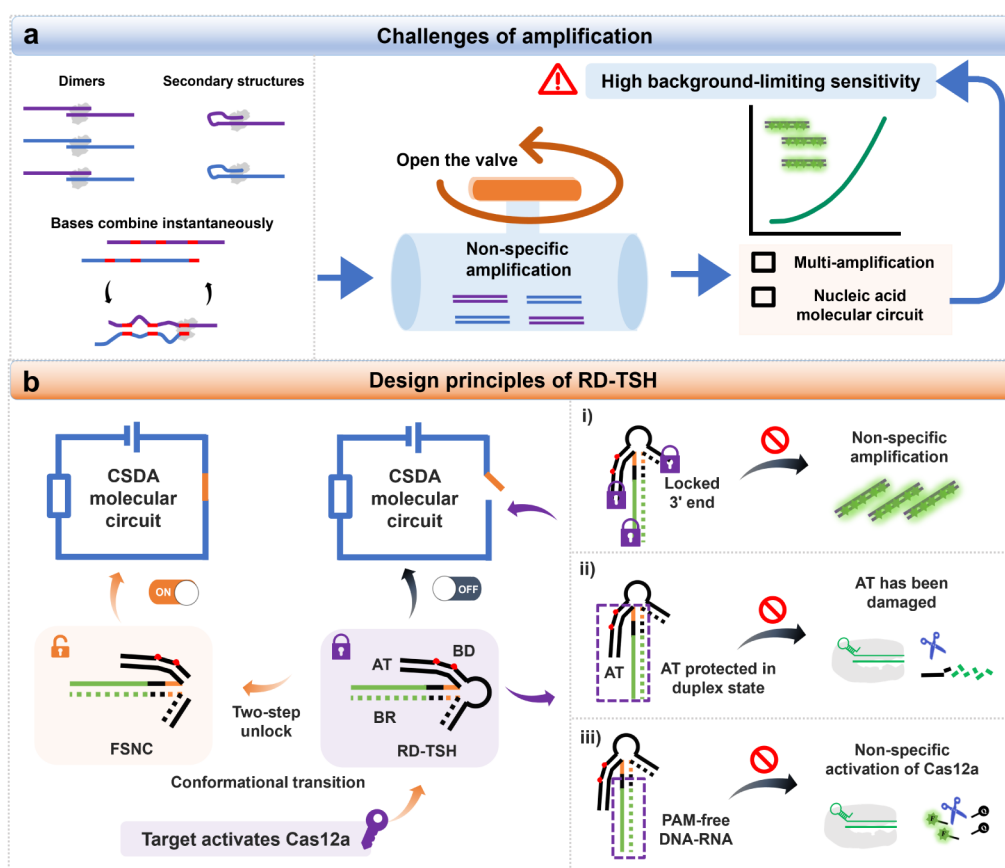


Figure 2. Design principles of RD-TSH nucleic acid switch. (a) Common challenges and limitations in isothermal amplification technologies. (b) Mechanism description of low-leakage and high amplification efficiency of RD-TSH in the CSDA system.

0". The SP and EP values were used to evaluate the initial unlocking efficiency of the RD-TSH0 to RD-TSH2 structures containing 0 to 2 base mismatches in the long branch domain.

FSNC O2, which retained two bases at the overhanging end of the long branch loop, was defined as the initial unlocked intermediate (Figure S3). In this structure, the base pairs were numbered from 1 to 8, starting with the first paired base at the 3' end of the long branch and proceeding toward the 5' end. The stability of the long branch ends in different FSNC O2 intermediates was assessed by calculating the hydrogen bond occupancy and root-mean-square fluctuation (RMSF) for the terminal 8 base pairs using oxDNA2. Finally, the hydrogen bond occupancy of the terminal base in each of the nine sticky ends (S0–S8) of FSNC2 (Figure S3) was calculated to further investigate the types of unlocked products capable of initiating SDA.

RESULTS AND DISCUSSION

Principle of CSDA Molecular Circuit with Self-Catalytic Positive Feedback

The molecular circuit-CSDA with self-catalytic positive feedback is designed for universal biosensing. This system can initiate triple cascade amplification and serves as a universal sensing module, enabling ultrasensitive detection of both nucleic acid and non-nucleic acid targets. The core of the CSDA circuit is a low-leakage nucleic acid molecular switch, RD-TSH, engineered to minimize nonspecific activation during SDA. By leveraging the high-turnover trans-cleavage activity of Cas12a toward ssDNA (Supplementary Table 1), the nuclease activity of Cas12a is activated by direct recognition of nucleic acid targets or, indirectly, by nucleic acid activators derived from non-nucleic acid targets, in which

Cas12a serves as the universal interface for CSDA (the first amplification stage, Figure 1a). This activation unlocks RD-TSH to generate the functional switch–nucleic acid complex (FSNC), which incorporates both primer and template sequences, thereby self-starting the inner SDA amplification cycle (the second amplification stage). The SDA amplification products act as Cas12a activators (C-activators), which induce Cas12a reactivation through positive feedback, triggering a new round of the “unlock–amplify” outer cycle (the third amplification stage). Owing to the sequence-independent trans-cleavage activity of activated Cas12a toward RD-TSH, the CSDA sensing module demonstrates a high degree of functional independence. To detect different targets, a target-specific pretreatment step is employed to generate the nucleic acid activator of Cas12a (Figure 1b). Simply selecting the crRNA corresponding to the target allows different nucleic acid activators to access the universal Cas12a interface, achieving two-step unlocking from RD-TSH to FSNC and gradually initiating the self-catalytic positive feedback of molecular circuit-CSDA: “unlocking–amplification–activation”, which generates target concentration-dependent fluorescence signals (Figure 1c). Notably, the high orthogonality (i.e., functional independence) between the pretreatment and sensing modules ensures the convenience and universality of the CSDA circuit for detecting diverse targets.

Design Principles of RD-TSH Nucleic Acid Switch

Under both classical Watson–Crick pairing and noncanonical base-pairing rules, isothermal amplification mixtures accumulate transient states, including template self-folding into

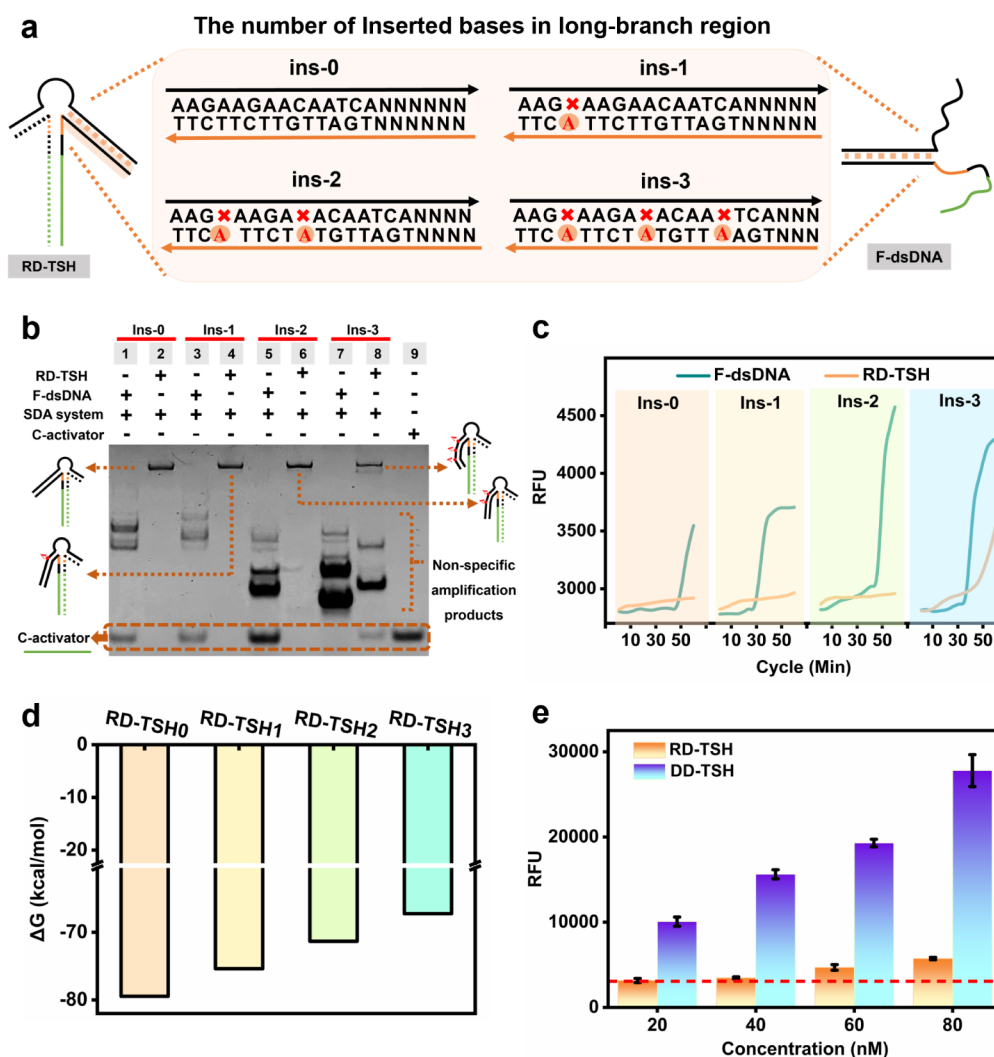


Figure 3. Background performance of RD-TSH and F-dsDNA switches in SDA and Cas12a systems. (a) Sequence schematics of RD-TSH and the corresponding F-dsDNA designs in the long-branch region. RD-TSH is assembled from BD, AT, and BR, whereas F-dsDNA is formed by partial duplexing of BD and AT. Variants differ by the number of inserted bases in the long-branch region (ins-0 to ins-3). (b) PAGE analysis and (c) real-time fluorescence curves of SDA background reactions using RD-TSH or F-dsDNA as amplification templates. (d) Binding free energies (ΔG) of the four RD-TSH variants. (e) Fluorescence responses of DD-TSH2 and RD-TSH2 in a single Cas12a reaction. DD-TSH2 is assembled by replacing BR with the DNA blocker BD# of the same sequence composition, together with BD2 and AT.

secondary structures or dimers and fleeting base pairing between template and primers. Such accumulation opens a “valve” for nonspecific amplification, thereby imposing high background signals on multi-amplification schemes and nucleic-acid molecular circuits and ultimately compromising sensitivity and specificity (Figure 2a).^{33–35} In principle, CSDA offers high sensitivity by engaging positive-feedback-driven triple-cascade amplification. Accordingly, stringent suppression of background leakage together with preserved amplification efficiency represents the key determinant of CSDA sensitivity. RD-TSH serves as the nucleic-acid switch in the positive-feedback circuit and therefore plays a central gatekeeping role. Hence, in the absence of target, two limiting requirements emerge for constructing an ultrasensitive CSDA positive-feedback circuit: RD-TSH must efficiently silence Cas12a, and the nonspecific amplification in the SDA pathway must be suppressed. Therefore, a dendritic RD-TSH is assembled by the amplification template (AT), a blocker DNA (BD), and a blocker RNA (BR). Partial complementarity among the three strands generates a central single-stranded loop protrusion and

a fully closed dendritic topology with three double-stranded arms (Figure S1a). This topological arrangement sequesters the 3' ends of three strands. In addition, each RD-TSH terminus remains locked in a more stable double-stranded conformation, which minimizes nonspecific amplification arising from random, transient base pairing at the 3' end of AT (Figure 2b, right (i)). In the same vein, BD and BR occlude all sites on AT, preventing AT from being disrupted by Cas12a trans-cleavage and thereby maintaining amplification efficiency for self-catalytic SDA (Figure 2b, (ii)). Crucially, taking advantage of the weak activation of Cas12a by RNA,^{4,36} a PAM-free BR is used to mask AT, further reducing the activation effect of the pre-existing activator sequence in the circuit on Cas12a (Figure 2b, (iii)). By strictly restraining nonspecific amplification and Cas12a self-activation, RD-TSH renders the target-induced conformational transition the sole trigger for CSDA initiation (Figure 2b, left), thereby establishing a circuit with low background and high amplification efficiency.

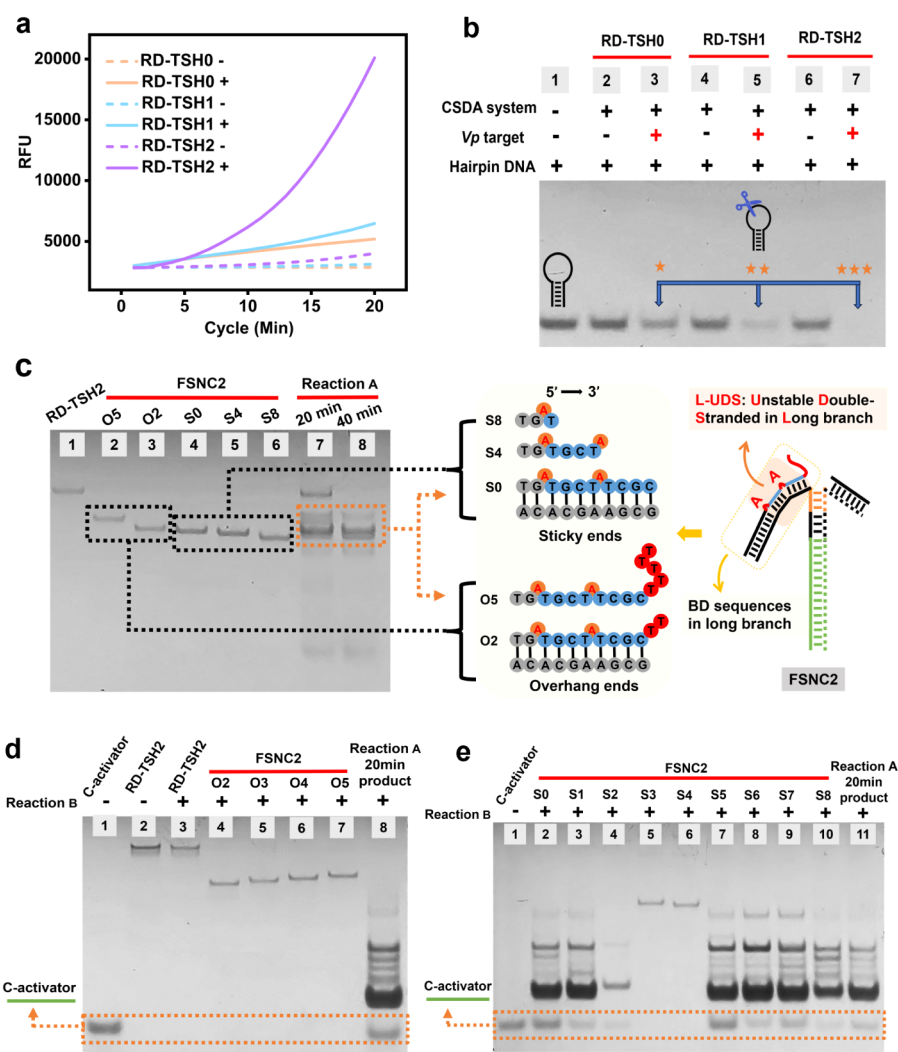


Figure 4. Feasibility and mechanistic investigation of the CSDA molecular circuit. (a) Real-time fluorescence curves and (b) 12% PAGE analysis of the CSDA circuits constructed using RD-TSH0 to RD-TSH2. (c) PAGE analysis of products generated upon Cas12a-mediated unlocking of RD-TSH2: lane 1, locked RD-TSH2; lanes 2–6, hypothesized and modeled conformations of the unlocking products (FSNC2); lanes 7–8, experimentally obtained unlocked products. (d) SDA efficiency comparison between overhang-end FSNC2 variants (O2–O5) and unlocking products. (e) SDA efficiency comparison between sticky-end FSNC2 variants (S0–S8) and unlocking products.

Engineering and Optimization of RD-TSH Nucleic Acid Switch

Following the design principles above, an optimal nucleic-acid switch combining low background leakage with high unlocking performance was achieved. Accordingly, 0 to 3 nucleotides were strategically inserted into the BD sequence within the long-branch region of RD-TSH. By tuning the rigidity of the loop single-stranded segment and the long-branch duplex, four switches with distinct DNA breathing fluctuation strengths were obtained, termed RD-TSH0, RD-TSH1, RD-TSH2, and RD-TSH3 (Figures 3a and S1a). 12% PAGE analysis confirmed successful construction of all four RD-TSH variants (Figure S1b). Their low background leakage was then assessed by comparing the SDA reaction with the Cas12a system.

First, four partially forked double-stranded DNA probes (F-dsDNA) matching the insertion numbers of the RD-TSH variants were designed (Figure 3a). SDA background reactions were performed using each RD-TSH together with its corresponding F-dsDNA, and nonspecific amplification backgrounds were characterized by PAGE analysis and real-time fluorescence testing (Figure 3b,c). Four incompletely sealed F-

dsDNA probes produced multiple nonspecific product bands (Figure 3b, lanes 1, 3, 5 and 7). In contrast, RD-TSH containing Ins-0, Ins-1, or Ins-2 generated neither the C-activator band nor other nonspecific bands (lanes 2, 4, and 6), whereas RD-TSH3 carrying Ins-3 yielded pronounced nonspecific bands in the SDA background reaction (lane 8). The fluorescence testing showed similar results with PAGE analysis. SDA reactions assembled from F-dsDNA exhibited substantially higher background fluorescence than those assembled from RD-TSH, and RD-TSH3 also produced a noticeable signal (Figure 3c). These data indicated that the fully sealed RD-TSH suppressed nonspecific amplification in SDA, relative to partially single-stranded F-dsDNA. Notably, RD-TSH3 still displayed background leakage despite having fully blocked 3' ends. This behavior likely resulted from excessive base insertion, which amplified thermodynamic DNA-breathing fluctuations and weakened duplex stability in the long-branch region, thereby exposing sites for nonspecific hybridization. To test this hypothesis, NUPACK was used to calculate the binding free energy (ΔG) of the four switches, which increased with the increasing number of inserted bases, consistent with

reduced switch stability (Figure 3d). In addition, the equilibrium-probability color map from NUPACK showed that the stability loss of RD-TSH3 mainly concentrated in the long-branch region containing the inserted bases (Figure S2).

In addition, efficient silencing of Cas12a by RD-TSH is required for achieving high specificity in the CSDA sensing system. Previous studies have reported that PAM-free dsDNA sequences do not activate Cas12a.^{4,26} Here, a blocker DNA# (BD#) containing a C-activator sequence without PAM was designed to replace BR for masking AT, thereby yielding a new DNA–DNA three-strand hairpin 2 (DD-TSH2) switch. This design enabled evaluation of the self-activation effects of RD-TSH2 and DD-TSH2 in a single Cas12a system. The PAM-free DD-TSH produced a clear activation response and the fluorescence intensity increased markedly with concentration (Figure 3e). Although this activation remained far weaker than that induced by conventional PAM-containing dsDNA targets, background signal became obvious when an activation sequence was embedded in a highly sensitive CSDA circuit as a high-concentration probe rather than as a low-abundance target. By contrast, when the RD-TSH concentration was below 40 nM, the fluorescence signal was nearly identical to the blank (Figure 3e), indicating negligible activation of Cas12a within this concentration range. Overall, these data further supported that the stable RD-TSH0, RD-TSH1, and RD-TSH2 led to strong locking effect in a CSDA circuit in the absence of target triggering.

Feasibility of CSDA Circuit and Mechanistic Insights

The RD-TSH could ensure low background interference while preserving high amplification efficiency upon unlocking. Notably, the real-time unlocking performance of target-activated Cas12a toward RD-TSH governed the initiation of molecular circuit-CSDA. Therefore, three strongly locking switches (RD-TSH0, RD-TSH1, and RD-TSH2), which were identified in the preliminary screening, were used to construct the corresponding CSDA circuits for the optimization of unlocking efficiency.

Using conserved nucleic-acid segment from the *Vp-thermolabile hemolysin* (*Vp-tlh*) gene in seafood as a dsDNA target (*Vp* target), the feasibility of sensing circuits built from three nucleic-acid switches was examined by real-time fluorescence curves and PAGE analysis, which showed the most pronounced signals of RD-TSH2 based CSDA circuit (Figure 4a,b). Using hairpin DNA as the reporter, little cleavage was observed in all three circuits in the absence of *Vp* target (lanes 2, 4, and 6). Upon addition of *Vp* target, the hairpin DNA bands became lighter in each circuit (lanes 3, 5, and 7), and the band disappeared completely in the RD-TSH2-based CSDA circuit (lane 7). Thus, RD-TSH2 was identified as the optimal switch combining low background leakage and high unlocking performance for constructing the CSDA molecular circuit.

Guided by the instability region of RD-TSH2 identified by NUPACK analysis (Figure S2) and the principles of the DNA-breathing,³⁷ the Cas12a cleavage site was hypothesized to reside within the single-stranded loop and the unstable double-stranded in long branch (L-UDS) region, and putative conformations of the unlocking products, denoted as FSNC2, were modeled (Figure S3). A fully complementary terminus in the long-branch BD segment was defined as the sticky end, whereas a terminus containing extra unpaired bases was defined as the overhang end. Accordingly, species were

named sticky 0 to sticky 8 (S0–S8) based on a 0–8 nt deficit at the 3' end of the BD strand, while the opposite configurations were termed overhang 2 to overhang 5 (O2 to O5) (Figures 4c and S3).

To clarify the unlocking process of RD-TSH2, the CSDA circuit was dissected into reactions A and B. To capture representative stages of the Cas12a-mediated cleavage process while preserving the intermediate unlocking products for PAGE analysis, incubation times of 20 and 40 min were selected for reaction A. Reaction A described target-activated Cas12a cleavage of RD-TSH2 that drove the conformational transition, and reaction B described SDA initiated by FSNC2. After incubation for 20 and 40 min, reaction A showed that the dominant product bands of RD-TSH2 (lanes 7 and 8, orange boxes) fell within the band range of FSNC2 from O5 to S8 (lanes 2 to 6, black boxes) (Figure 4c). A time-resolved comparison further revealed that the 20 min incubation produced broad band, indicating that Cas12a cleavage of RD-TSH2 produced a mixture rather than a single species. When the reaction time was extended to 40 min, slower-migrating band became weaker, while the position of the major unlocking-product band remained essentially unchanged, and the original RD-TSH2 band disappeared (Figure 4c, lane 8), suggesting complete disruption of the intact conformation. Therefore, the working hypothesis was proposed as Figure S4. At the early stage, Cas12a initiated SDA through a two-step unlocking of RD-TSH2. In unlock 1, Cas12a cleaved the loop of RD-TSH2 at random positions, generating FSNC2 O2 to O5 species bearing an overhang end. Subsequently, the L-UDS region of FSNC2 O2 to O5 underwent thermally induced DNA 3' end breathing, and the exposed ssDNA was captured and cleaved by Cas12a, yielding FSNC2 S0 to S8 with a sticky end. This process constituted unlock 2, corresponding to full unlocking.

According to the two-step unlocking hypothesis, FSNC2 O2–O5, FSNC2 S0–S8, and the 20 min products from reaction A were used in reaction B to assess the self-priming amplification efficiency of the 13 modeled unlocking products as well as the experimental products of the SDA system (Figure 4d,e). PAGE analysis showed that the modeled unlock-1 product, FSNC2 O2–O5, did not self-initiate SDA (Figure 4d, lanes 4–7). For the unlock-2 products, all FSNC2 except S3 and S4 (Figure 4e, lanes 5 and 7) triggered SDA (Figure 4e, lanes 2–4 and 7–11), generating the C-activator that initiated the positive-feedback loop (Figure 4d,e, orange boxes). These findings demonstrated that only the products of both unlocking steps were competent to enter the self-initiating SDA program. Notably, the C-activator bands produced by FSNC2 S0–S8 in reaction A exhibited varying intensities, suggesting that distinct FSNC2 conformations led to differential amplification efficiencies, likely attributable to variations in the stability of base-pairing at the 3' ends. Specifically, FSNC2 S3 and S4 failed to initiate SDA, likely because the two mismatched bases introduced into the long branch were positioned close to the 3' termini, disrupting the stable base-pairing required for self-priming initiation and thereby reducing or abolishing amplification efficiency. The structural basis underlying these differences was further investigated by MD simulations in the following section. Furthermore, the experimental unlocking products derived from reaction A were also shown to activate the SDA program, specifically generating the C-activator. The amplification bands closely matched those produced by the modeled products (Figure 4d, lane 8; Figure

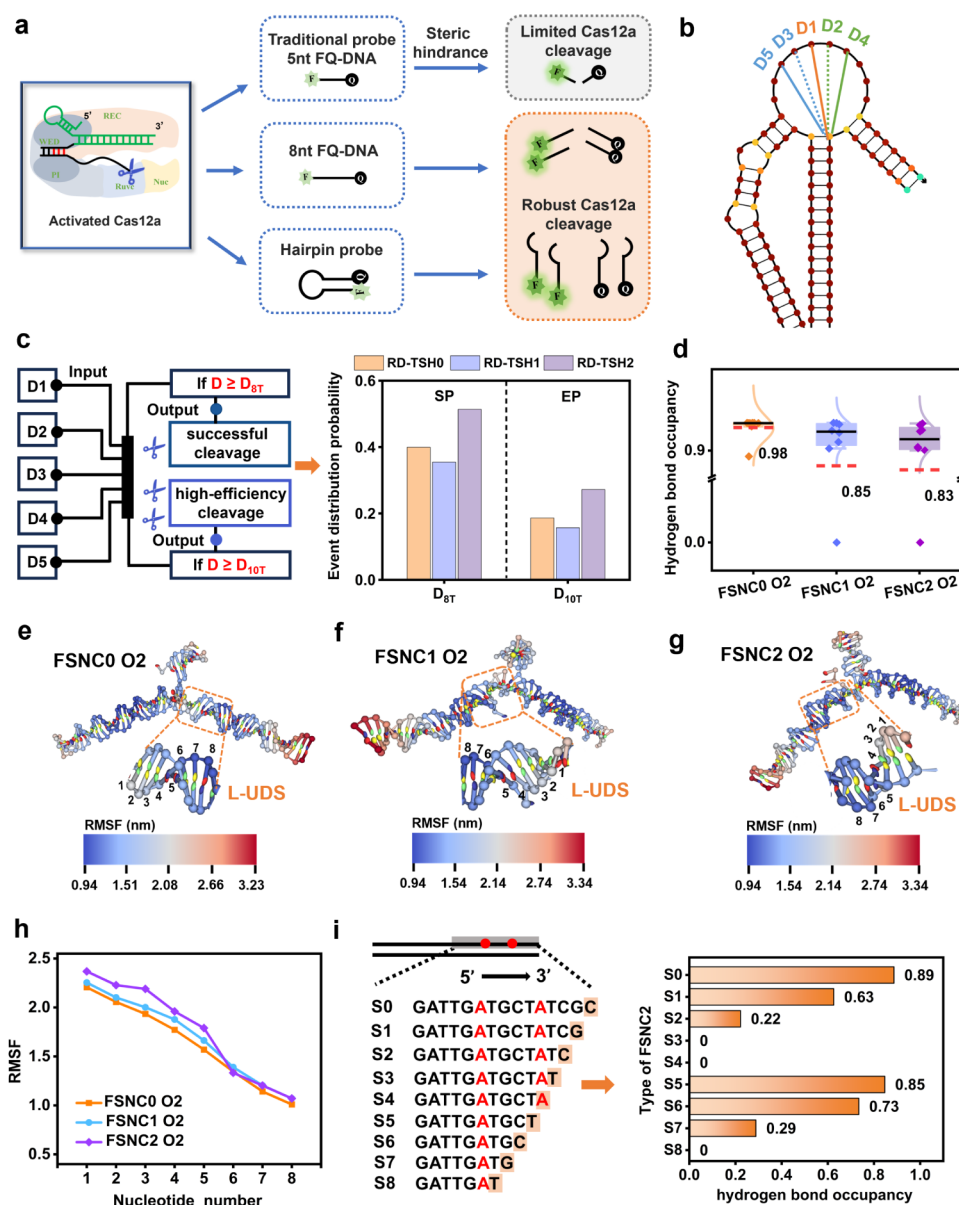


Figure 5. MD simulations of “two-step unlocking” and “self-initiated amplification” mechanisms in the positive-feedback molecular circuit-CSDA. (a) Schematic comparison of Cas12a cleavage efficiencies for different reporter formats. (b) Definition of the loop spatial-distance parameters (D1–D5) of RD-TSH. (c) Decision models for the probability of successful cleavage events (SP) and efficient cleavage events (EP) for RD-TSH0–RD-TSH2. Left, workflow for classifying successful and efficient cleavage; right, grouped bar plots of SP and EP for RD-TSH0–RD-TSH2. (d) Hydrogen-bond occupancy of the eight base pairs in L-UDS for the step-1 modeled unlocking products of the three nucleic-acid switches. The red line denotes the mean (value labeled) and the black line denotes the median. (e–g) centroid structural models of the three modeled unlocking products. (h) RMSF of the eight base pairs in L-UDS, elucidating “breathing” of base pairs at the 3' end and the associated structural stability after Cas12a triggering. (i) Sequences of FSNC2 S0–S8 in L-UDS (left) and hydrogen-bond occupancy of the terminal base at the L-UDS 3' end, interrogating unlocking-product conformations competent to initiate isothermal amplification (right). All data were obtained from oxDNA MD simulations followed by quantitative analyses.

4e, lane 11). These results provided additional support for the two-step unlocking hypothesis and confirmed the structural accuracy of the proposed conformations for the modeled unlocking products.

MD Simulation

To further elucidate the molecular mechanism underlying CSDA operation, MD simulations of RD-TSH and FSNC were performed using the oxDNA2 model to identify key parameters governing the efficiencies of the “two-step unlocking” process and the “self-initiated amplification” module. Although a 5-nt fluorophore/quencher-labeled

ssDNA (FQ-ssDNA) reporter has been widely used in CRISPR–Cas12a assays,^{38,39} the steric constraints between Cas12a and the reporter labels limited trans-cleavage, and substantially higher trans-cleavage activity was observed toward ≥ 8 -nt linear ssDNA reporters or hairpin DNA reporters (Figure 5a).^{40–43} Thus, Cas12a activated by a defined target sequence was evaluated with reporter molecules of distinct lengths and conformations, including linear ssDNA with 5-, 8-, and 10-nt, as well as hairpin DNA reporter molecules. Consistent with prior findings, the cleavage efficiency followed

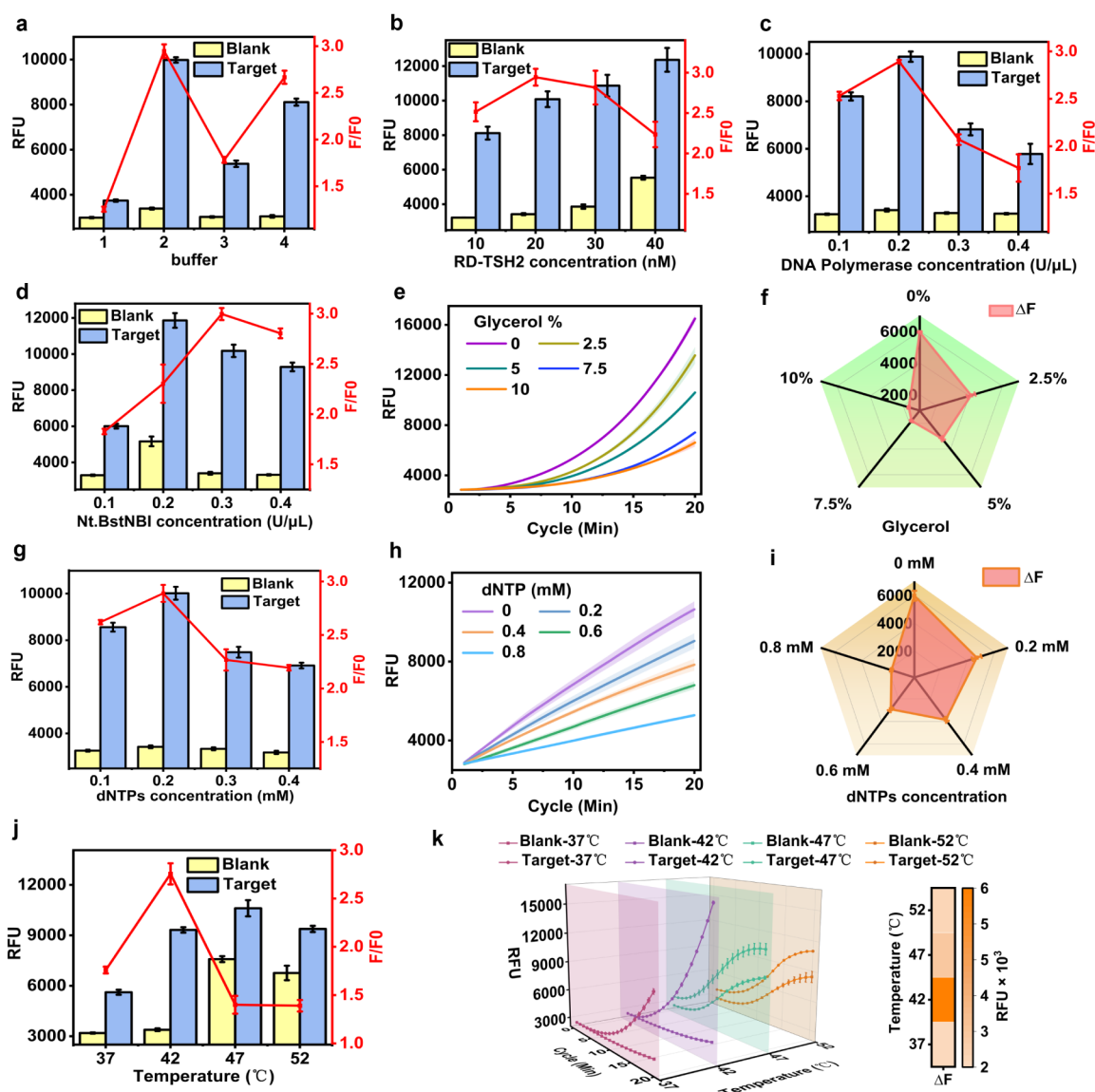


Figure 6. Key parameter optimization of the CSDA sensing module. (a) Reaction buffer type; (b) RD-TSH2 concentration; (c) DNA polymerase concentration; (d) Nt.BstNBI concentration; (g) dNTP concentration and (j) reaction temperature. Error bars represent the mean $\pm$ SD from three replicates. Blank denotes CSDA without target. (e) Real-time fluorescence curve and (f) glycerol concentration radar plot of CSDA sensing at varying glycerol levels (1 pM *Vp-*tlh**). (h) Real-time fluorescence curve and (i) dNTP concentration radar plot of Cas12a sensing at varying dNTP levels (1 nM *Vp-*tlh**). (k) Real-time fluorescence curve (left) and temperature heatmap (right) of CSDA at different reaction temperatures. Radar plots and heatmaps were generated using ΔF , defined as the fluorescence intensity difference at 15 min between target absent and target present reactions.

the order: hairpin DNA $\approx$ 10-nt ssDNA > 8-nt ssDNA > 5-nt ssDNA (Figure S5).

The spatial distance within the single-stranded loop of RD-TSH constituted a decisive factor that limited the efficiency of the first-step unlocking. The distances between the top five nucleotides in the RD-TSH loop and the nucleotide at the inflection point of the main trunk were defined as key structural parameters, denoted D1–D5, to evaluate the first step unlocking efficiency of Cas12a for the three RD-TSH designs (Figure S5b). D_{8T} and D_{10T} were defined as the criteria for “successful cleavage” and “efficient cleavage” according to prior studies^{40,41} and the experimental observations, respectively (Figure S4), where D_{8T} and D_{10T} represented the end-to-end base distances of 8-nt and 10-nt ssDNA reporters. To eliminate the discrepancy in reporter length between the oxDNA2 model and the physical state, oxDNA2 was used to

simulate the dynamic lengths of 8-nt ssDNA and 10-nt ssDNA reporters, and the mean values of D_{8T} and D_{10T} were 2.49 and 2.94 nm, respectively (Figure S6). Next, D1–D5 distance parameters were extracted from all oxDNA trajectories of three RD-TSH variants, and a two-dimensional code map was generated by combining a heatmap of loop spatial distances with a heatmap of cleavage-event distributions (Supplementary Figures 7, 8). RD-TSH2 exhibited substantially larger loop spatial distances than RD-TSH0 and RD-TSH1 (the highest fraction of orange pixels), indicating the most favorable Cas12a trans-cleavage activity toward RD-TSH2. Using D_{8T} and D_{10T} as decision values, the models were established to classify successful cleavage ($D \geq D_{8T}$) and efficient cleavage ($D \geq D_{10T}$) (Figures S5c, S7 and S8), and the probabilities of successful and efficient cleavage events were calculated using eqs 1 and 2, yielding SP and EP, respectively. These analyses

indicated that RD-TSH2 achieved a markedly higher unlock 1 efficiency than the other two switches, with the performance ranked as RD-TSH2 > RD-TSH0 > RD-TSH1 (Figure 5c, right). Notably, the unlock 1 ranking was not fully consistent with the experimentally observed amplification efficiency of CSDA (RD-TSH2 > RD-TSH1 > RD-TSH0) (Figure 4a,b), suggesting that the correlation between “two-step unlocking” and “self-initiated amplification” warranted further investigation.

The PAGE analysis revealed that only seven unlock 2 species bearing a sticky end initiated the SDA program among the 13 modeled unlocking products (Figure 4d,e), indicating that unlock 2 was required for entry into the self-initiated amplification module. Under the reaction condition of 42 °C, FSNC2 O2–O5 (unlock 1 products) exhibited pronounced breathing fluctuations at the 3' end because two inserted bases were present in the L-UDS region. This effect exposed ssDNA sites accessible to Cas12a and subsequently generated the unlock 2 products FSNC2 S0–S8. To evaluate this inference, MD simulations were performed on three representative unlock 1 products from the RD-TSH designs (FSNC0 O2 to FSNC2 O2) to analyze the hydrogen-bond occupancy and root-mean-square fluctuation (RMSF) of the eight base pairs within the L-UDS region. With increasing of mismatch, the hydrogen-bond occupancy within L-UDS decreased progressively (Figure 5d). The value below 1 indicated hydrogen bond rupture in the corresponding base pair. In the centroid structural models of three unlocking products, the RMSF of the L-UDS region increased stepwise (Figure 5e–g), and the RMSF differences were primarily concentrated within the first five nucleotides (Figure 5h), indicating that this segment exhibited more active thermodynamic fluctuations than the remaining regions.

Therefore, introducing mismatch into the long branch of RD-TSH compromised duplex stability and induced pronounced breathing fluctuations of base pairs within the L-UDS region of FSNC. The inferred fluctuation intensity of the three unlock 1 products followed the order: FSNC2 O2 > FSNC1 O2 > FSNC0 O2, consistent with the experimentally observed ranking of CSDA sensing signal intensities mediated by the three RD-TSH designs (Figure 4a,b). This agreement supported that unlock 2 constituted the rate-limiting step for initiating the molecular circuit-CSDA. Given the distinct SDA initiation efficiencies observed among the nine modeled unlock 2 products FSNC2 S0–S8 (Figure 4e), MD simulations were performed for FSNC2 S0–S8 to evaluate the hydrogen-bond occupancy of the terminal base at the 3' end of L-UDS. FSNC2 S3, S4, and S8 displayed zero hydrogen bond occupancy (Figure 5i), indicating persistent bond disruption, consequently, failure to initiate amplification. In addition, hydrogen-bond occupancy increased with the increasing distance from the inserted “A” base, reflecting enhanced local stability. These results indicated that the amplification efficiency of the nine species in the SDA reaction correlated with sequence stability at the 3' end of L-UDS.

Optimization of CSDA Sensing Module

To maximize the performance of the CSDA sensing module, the fluorescence intensity ratio in the presence to absence of 10 pM target (F/F₀) was used to optimize the routine parameters. The Cas12a concentration, Cas12a-to-crRNA concentration ratio, FQ-DNA reporter concentration, and

preincubation time between Cas12a and target were optimized to be 40 nM, 1:1, 2 μM, and 15 min (Figure S9), respectively.

Four reaction buffers were formulated according to the recommended buffers for three enzymes involved in CSDA (Supplementary Table 2), and buffer 2 provided the best compatibility among all enzymes (Figure 6a). RD-TSH2 functioned as the pivotal nucleic acid switch in the CSDA positive feedback circuit. An optimal balance between low leakage and high amplification (F/F₀) was identified at 20 nM (Figure 6b). Increasing the concentrations of the DNA polymerase and the nicking endonuclease Nt.BstNBI to 0.2 U/μL maximized CSDA efficiency (Figure 6c,d). Notably, the enzyme concentrations above 0.2 U/μL led to a pronounced decline in reaction efficiency. Examination of the storage buffer formulations for three enzymes revealed high glycerol contents (Supplementary Table 3). Accordingly, glycerol was titrated into the CSDA sensing system at 0%, 2.5%, 5%, 7.5%, and 10%, and the fluorescence intensity decreased progressively as the glycerol concentration increased (Figure 6e,f). This reduction likely reflected slower SDA kinetics at elevated glycerol levels⁴⁴ and phase separation among reaction components at the beginning of the reaction due to increased viscosity.^{18,45} At lower enzyme loadings, enzymatic enhancement dominated and accelerated the reaction, whereas once the gain approached a plateau or increased more slowly, further increase in enzyme concentration introduced additional glycerol from the storage buffer, strengthening inhibition and reducing efficiency. Therefore, an enzyme concentration of 0.2 U/μL established the optimal balance between enzymatic enhancement and glycerol induced inhibition, giving the highest reaction rate.

Sufficient dNTP concentrations are widely recognized as essential for sustaining high DNA polymerase reaction rates.^{46,47} However, the reaction rate dropped sharply in the CSDA system, when dNTPs exceeded 0.2 mM (Figure 6g), which aligned with the inhibitory effect of dNTPs on Cas12a observed in separate activity assays (Figure 6h,i). At low dNTP concentrations, the enhancement of polymerase activity dominated the overall behavior. With the increasing dNTP concentration, the balance shifted from polymerase enhancement toward suppression of Cas12a activity. Therefore, 0.2 mM dNTPs brought the antagonistic contributions of polymerase enhancement and Cas12a inhibition into equilibrium in the CSDA system. The recommended reaction temperature in the manual for the DNA polymerase and Nt.BstNBI used in the CSDA sensing module are 55 °C. This value differed substantially from the recommended range for Cas12a (25–48 °C), making temperature optimization critical for improving CSDA sensing performance. The highest F/F₀ occurred at 42 °C (Figure 6j,k). Excessive temperature reduced RD-TSH2 stability and even disrupted the dendritic conformation (Figure S10), leading to severe background leakage.

Performance of CSDA Mediated Nucleic Acid Analysis

Vp has been recognized as a high risk marine foodborne pathogen.^{42,48} The gene encoding its key virulence factor, thermostable direct hemolysin (TLH), was selected as the target to compare the analytical performance of the CSDA sensing module with a standalone Cas12a assay. The linear range for Vp DNA spanned 1 pM–10 nM in the standalone Cas12a system and 10 aM–10 pM in CSDA sensing. The limits of detection (LOD), calculated using 3 times standard

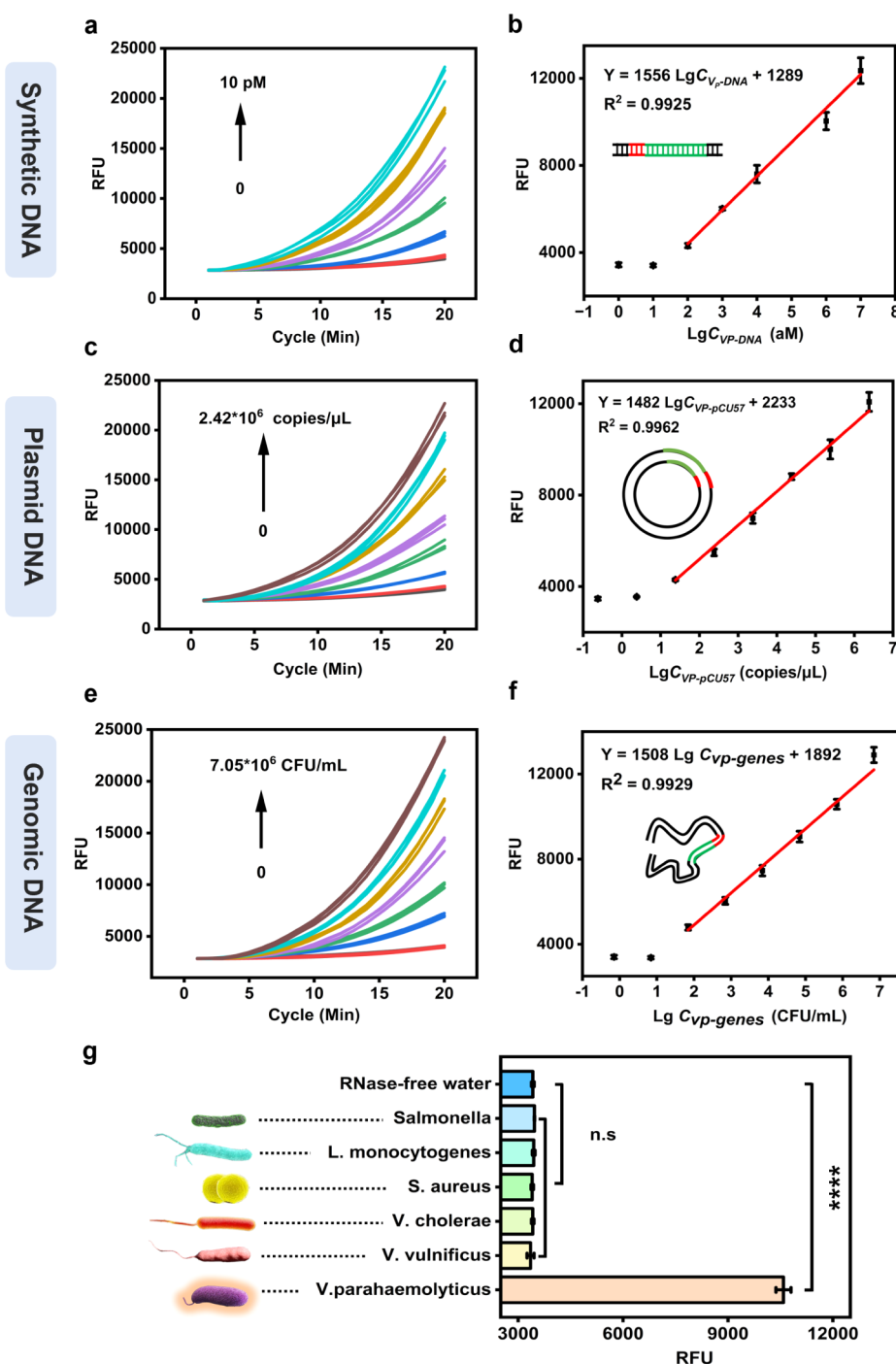


Figure 7. Sensitivity and specificity of CSDA sensing toward *Vp-tlh* nucleic acid. (a, c, e) Fluorescence amplification curves and (b, d, f) corresponding calibration curves for synthetic DNA containing *Vp-tlh* sequence (a, b), circular plasmid *Vp-pCUS7* containing the *Vp-tlh* sequence (c, d), and *Vp* genomic DNA extracted directly from *Vp* cultures (e, f). (g) Specificity analysis of the CSDA sensing module against genomic DNAs from six foodborne pathogens. The target concentration was 7.05×10^6 CFU/mL, and RNase-free water served as control. Statistical analysis was performed using one-way analysis of variance (ANOVA). n.s. indicates not significant ($P > 0.05$) and **** indicates $P < 0.0001$. Data are presented as mean $\pm$ SD ($n = 3$ technical replicates).

deviations (eq S1), were 492.06 fM and 4.20 aM, respectively (Figures S11 and 7a,b). The positive feedback CSDA cascade reduced the LOD by 5 orders of magnitude.

A strong linear relationship was observed between the logarithm of target concentration and the fluorescence signal for all three targets (Figure 7b,d,f). The calibration curves for *Vp* DNA, *pCUS7*, and *Vp* genomic DNA were $Y = 1556 \text{ Lg} C_{VP-DNA} + 1289$ ($R^2 = 0.9925$), $Y = 1482 \text{ Lg} C_{pCUS7} + 2233$

($R^2 = 0.9962$), and $Y = 1508 \text{ Lg} C_{vp\text{-}genes} + 1892$ ($R^2 = 0.9929$), yielding the LODs of 4.20 aM, 9.40 copies/ μL , and 14.79 CFU/mL, respectively. Overall, CSDA exhibited high sensitivity across all the tested targets. Robust detection was obtained not only for short synthetic sequences but also for more complex long nucleic acid targets, including plasmids and genomic DNA. Its performance was superior to or comparable with those of several recent studies (Supplementary Table 4).

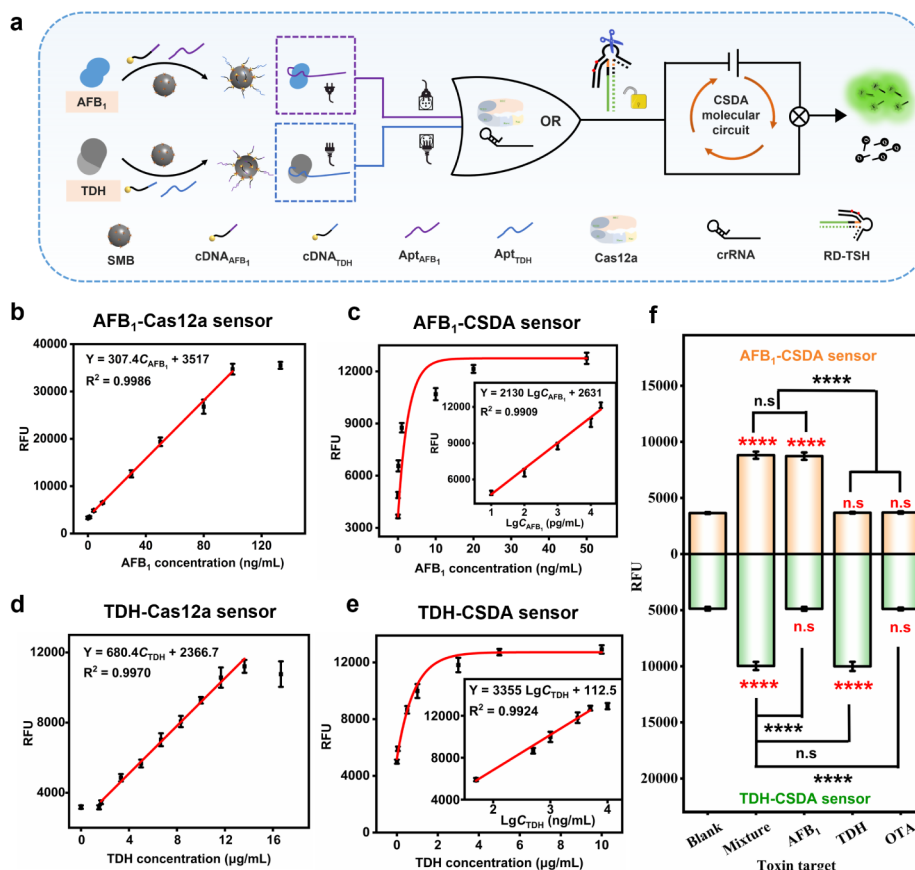


Figure 8. Sensitivity and specificity analysis of CSDA sensing module for the small molecule targets AFB₁ and TDH. (a) Schematic illustration of nucleic acid conversion of small molecule targets followed by CSDA sensing. Calibration curves for AFB₁ detection in (b) standalone Cas12a sensing and (c) CSDA sensing. Calibration curves for TDH detection in (d) standalone Cas12a sensing and (e) CSDA sensing. (f) Specificity analysis of CSDA sensors for AFB₁ (top) and TDH (bottom). The concentrations of AFB₁, TDH, and OTA were 1 ng/mL, 1 μg/mL, and 1 ng/mL, respectively. Mixtures contained AFB₁, TDH, and OTA. RNase-free water served as a blank reaction. One-way analysis of variance (ANOVA) was used to compare fluorescence signals between each small molecule group and the blank (red) and between different small molecule groups (black). n.s. indicates not significant ($P > 0.05$) and **** indicates $P < 0.0001$. Data are presented as mean $\pm$ SD ($n = 3$ technical replicates).

Specificity was further assessed against six foodborne pathogens, which indicated that the CSDA sensor displayed excellent specificity for *Vp* (Figure 7g).

In addition, the detection sensitivity of CSDA was systematically evaluated using three target types, including synthetic *Vp* DNA, plasmid pCU57, and *Vp* genomic DNA extracted directly from bacteria. Unlike the amplification profiles reported for several CRISPR Cas12a cascade systems,^{49,50} the real-time fluorescence curves for all three targets exhibited an approximately 10 min slow growth phase (Figure 7a,c,e). Segmented kinetic analysis was performed on the real-time fluorescence curves obtained at each target concentration. Linear regression was conducted over the intervals of 6–10 min and 11–15 min, and the resulting slopes were defined as the apparent reaction rates v_{6-10} and v_{11-15} , respectively. A pronounced increase in CSDA sensing rate emerged at approximately 10 min for all three targets, with a more prominent rise observed at higher target concentrations (Figure S12). Thus, it could be inferred that the inserted bases drove transient strand dissociation and rehybridization, which dominated the early phase with inefficient amplification. Consequently, the fully unlocked product FSNC2 exhibited reduced amplification efficiency at the beginning of the reaction. As the C-activator accumulated, Cas12a activation initiated additional rounds of the CSDA circuit. Positive

feedback amplification then became dominant, increasing the overall reaction efficiency. These results indicated that CSDA operated through autocatalytic Cas12a activation driven by internally accumulated C-activator, which in turn enabled positive feedback cascade amplification.

Performance of CSDA Mediated Non-Nucleic Acid Target Analysis

The secondary metabolite aflatoxin B₁ (AFB₁), generated by aspergillus species, is a thermally stable contaminant of key crops. It is recognized for its carcinogenic, mutagenic, and teratogenic potency.⁵¹ Thermostable direct hemolysin (TDH) is a key pathogenic factor of *Vp*, possessing hemolytic activity, cytotoxicity, enterotoxigenicity, and lethality.⁵² Here, the pretreatment modules corresponding to AFB₁ and TDH were optimized (Supplementary Figures 13a,b and 14a–c) to evaluate the performance of CSDA sensing module for non-nucleic acid targets (Figure 8a). Under optimal conditions, both the single Cas12a assay and the CSDA method enabled quantitative detection of AFB₁ and TDH, and the latter demonstrated markedly enhanced sensitivity. For AFB₁, the single Cas12a assay exhibited a linear range of 4 to 100 ng/mL and a LOD of 2.57 ng/mL at 3SD (Figure 8b). In contrast, the CSDA method achieved a linear range from 0.01 to 20 ng/mL and a LOD of 4.27 pg/mL, corresponding to a 602-fold improvement in sensitivity (Figure 8c). For TDH, the single

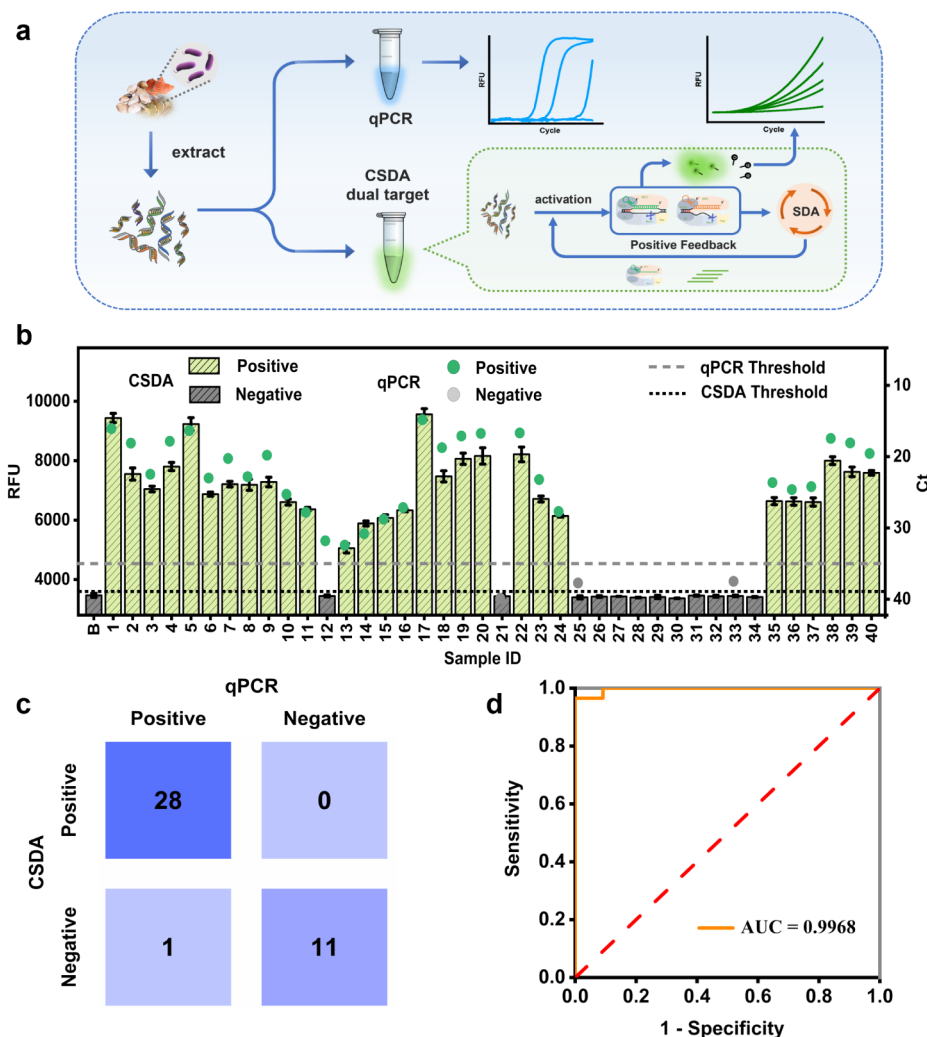


Figure 9. Practical application of CSDA sensing module for *Vp-tlh* nucleic acid and non-nucleic acid targets AFB₁ and TDH. (a) Schematic illustration of *Vp-tlh* testing in real samples by qPCR and the CSDA assay. (b) *Vp-tlh* detection in 40 seafood samples with CSDA method and qPCR. The sample set comprised 10 shrimps (1–10), 10 *Linnaeus* (11–20), 14 oysters (21–34), and 6 scallops (35–40). “B” denotes RNase free water as negative control. qPCR served as the reference method, classifying 29 samples as *Vp* positive (green) and 11 samples as *Vp* negative (gray). The qPCR threshold (long dashed line) was defined by the negative standard of the qPCR kit ($C_t = 35$). The CSDA threshold (short dashed line) was defined as the maximum fluorescence of the negative controls plus 3 times standard deviation (RFU = 3537). (c) Confusion matrix and (d) receiver operating characteristic (ROC) curve analyses to compare the accuracy and specificity of CSDA and qPCR for discriminating *Vp* positive and *Vp* negative samples.

Cas12a assay showed a linear response from 1.67 to 13.63 $\mu\text{g/mL}$ with a LOD of 1.62 $\mu\text{g/mL}$ (Figure 8d), whereas the CSDA method provided a linear range of 0.05 $\mu\text{g/mL}$ to 5 $\mu\text{g/mL}$ and a LOD of 38.43 ng/mL, representing a 42-fold enhancement in sensitivity (Figure 8e). Collectively, the CSDA sensing module provided substantially broader linear ranges and lower detection limits for both analytes compared to the single Cas12a reaction. Notably, the improvement in sensitivity of the CSDA method for AFB₁ (602-fold) was substantially greater than that for TDH (42-fold). This discrepancy was primarily attributed to the difference in aptamer performance. AFB₁, a small-molecule toxin that has long been a major target for monitoring, benefits from well-established aptamers with exceptionally high affinity and specificity, which enables more efficient initiation of the subsequent signal amplification cascade. In contrast, the binding performance of the aptamer for the TDH protein is likely more limited, resulting in a

comparatively lower overall enhancement of detection sensitivity.

Subsequently, the specificity analyses of AFB₁, TDH, Ochratoxin A (OTA) and their mixtures were conducted in AFB₁–CSDA and TDH–CSDA sensing. Only the specific targets and related mixture showed significantly different fluorescence signals compared to the blank control ($p < 0.001$), while the presence of other nontarget small molecules in the mixtures did not interfere with the signal, confirming the excellent specificity and anti-interference capability of the sensing method.

Assessment of CSDA Sensing Performance in Practical Samples

Given the excellent analytical performance of the proposed autocatalytic CSDA assay for both nucleic acid and non-nucleic acid targets, its practical applicability was further evaluated for detecting the *Vp-tlh* gene and quantifying TDH in seafood and AFB₁ in peanuts. Genomic extracts from 40

randomly seafood samples were analyzed for *Vp* using both qPCR and the CSDA assay (Figure 9a). qPCR served as the reference method, and samples with a cycle threshold (*Ct*) value ≤ 35 were defined as positive. Using this criterion, 29 positive and 11 negative samples were identified (Figures 9b and S15–18). The CSDA assay correctly classified all 11 negative samples and 28 of the 29 positive samples, with one false negative observed for a sample with a high *Ct* value (Sample 13) (Figure 9b). A confusion matrix and receiver operating characteristic (ROC) curve were subsequently generated (Figure 9c,d). The area under the curve (AUC) reached 0.9968, indicating strong agreement with qPCR outcomes. Accordingly, the CSDA assay achieved 100% specificity and 96.55% sensitivity for *Vp* detection in real seafood samples. The detection capability of CSDA in real samples was comparable to that of qPCR. Practicality was improved by isothermal operation at 42 °C, a shorter assay time (30 min), and elimination of thermal cycling required for qPCR (55 min). The practical utility of CSDA for non-nucleic acid targets was further validated by spiking and recovery tests for AFB₁ in peanuts and TDH in shrimps (Supplementary Tables 5, 6). The method showed satisfactory accuracy and precision, with recovery of 91.05% to 105.11% for AFB₁ in peanut samples and 86.29% to 105.39% for TDH in shrimp samples. The relative standard deviations (RSD) were below 11% in all cases. Overall, these results demonstrated high sensitivity, rapid response, operational simplicity, and broad applicability of the proposed CSDA assay methods across nucleic acid and non-nucleic acid targets, supporting their practical applications in on site food safety monitoring and point-of-care diagnostics.

CONCLUSIONS

CRISPR/Cas12a has emerged as a revolutionary tool for molecular diagnostics due to its high specificity. Its practical application remains constrained by two major challenges: 1) limited sensitivity, with preamplification-dependent strategies introducing aerosol contamination risks,^{10–12} 2) inherent target restrictions due to the native recognition mechanism, hindering simultaneous detection of broader biomarkers such as proteins and small molecules.^{14,21–23} To overcome these limitations, this study develops an RD-TSH nucleic acid switch with low background leakage and high amplification efficiency, systematically elucidating its working mechanism for low-background leakage, two-step unlocking, and product-activated SDA, and constructs a preamplification-free and autocatalytic molecular circuit-CSDA. We further demonstrated that CSDA circuit performance is governed by the structural stability of RD-TSH and the 3'-end breathing fluctuations of its unlocked products, providing new insights and strategies for the tailored design of sensing molecular circuits. This integrated strategy directly embeds signal amplification within the Cas12a reaction system to eliminate contamination risks from separate amplification steps, and employs an efficient positive-feedback loop to achieve triple cascade amplification via “unlocking–amplification–activation,” which elevates the detection sensitivity to aM level. Benefiting from the high programmability of the Cas12a-crRNA complex, the CSDA circuit can act as an independent and universal sensing module and be flexibly coupled with different upstream preprocessing modules by simply replacing the crRNA for both nucleic acid and non-nucleic acid targets. The superior sensitivity, specificity, and generalizability of the molecular circuit-CSDA have been

systematically validated through the detection of *Vp* nucleic acids, AFB₁, and TDH. Its practical potential has also been demonstrated by successful application in real seafood and peanut samples, underscoring its strong utility for food safety monitoring.

While the CSDA assay demonstrates excellent performance, there remains room for improvement in its direct detection efficiency for long genomic targets. This may be attributed to two factors: long DNA strands may exert an inhibitory effect on the Cas12a nuclease activity,⁵³ and the presence of abundant nonspecific PAM sites in genomic sequences may interfere with and delay the reaction kinetics. Future efforts may further enhance system activity through strategies such as engineered crRNAs^{54,55} or the addition of reaction enhancers.^{56,57} The high versatility of this platform enables multidimensional bioanalysis. For instance, simultaneous detection of pathogen nucleic acid and their secreted toxin proteins allows for more accurate biological risk assessment. With the development of additional high affinity recognition elements, the platform may be further expanded into a multiplex detection array. When integrated with microfluidics, portable devices, and machine learning algorithms, it holds promise for evolving into an intelligent diagnostic system capable of rapid detection, strain typing, and risk warning, thereby advancing CRISPR based sensing from a detection tool toward a comprehensive analytical platform.

ASSOCIATED CONTENT

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.6c04985>.

Materials; treatments of nucleic acids; nucleic acid targets and non-nucleic acid targets; single CRISPR/Cas12a reaction; single SDA reaction; NUPACK analysis; detection of *Vp* in real samples; detection of AFB₁ and TDH; calculation of LOD; supplementary figures (Figures S1–S18) and supplementary tables (Tables S1–S4); additional references cited by authors (PDF)

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Notes

The authors declare no competing financial interest.

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