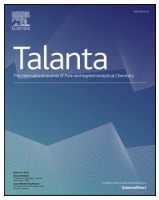
Talanta 288 (2025) 127730



Contents lists available at ScienceDirect

Talanta

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Low-background CRISPR/Cas12a sensing system with circular CRISPR RNA for amplified fluorescent detection of antibody in human serum

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ARTICLE INFO

Keywords:
Anti-dig antibodies
CRISPR/Cas12a
Circular CRISPR RNAs
Fluorescence
Low-background

ABSTRACT

Regular monitoring of serum antibody levels is crucial for preventing interference with therapeutic effectiveness and reducing the risk of toxicity. To address this, a CRISPR/Cas12a sensing system with circular CRISPR RNAs (^{C}crRNAs) is described for highly sensitive detection of anti-digoxin (Anti-Dig) antibodies in human serum. In this work, the topology structure of ^{C}crRNAs effectively suppresses the function of linear crRNAs (^{L}crRNAs), making them unable to regulate the *cis-/trans*-cleavage activity of the Cas12a system. Therefore, a low-background is obtained in the absence of targets. The target Anti-Dig antibodies trigger the assembly of the complete multicomponent nucleic acid enzyme (MNAzyme) with active enzyme activity, which can transform ^{C}crRNAs into ^{L}crRNAs. The ^{L}crRNAs further recover the *trans*-cleavage activity of the CRISPR/Cas12a system, which can degrade single-stranded reporter DNA to generate a significantly enhanced fluorescent signal. This method enables sensitive detection of Anti-Dig antibodies as low as 15 pM within 60 min and exhibits a linear detection range of 25 pM–50 nM. It also exhibits excellent selectivity against non-target antibodies and has been successfully validated in diluted serum samples, achieving a recovery rate ranging from 96.16 % to 103.08 %. This novel CRISPR/Cas12a sensing system with ^{C}crRNA represents a powerful and efficient tool for detecting low-abundance biomarkers in complex biological samples.

1. Introduction

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system is an adaptive immune mechanism found in bacteria and archaea, providing defense against invading viral DNA [1,2]. This system comprises CRISPR sequences and associated Cas proteins, with Cas proteins playing a pivotal role in cleaving nucleic acids. Among these proteins, Cas12a (Cpf1) is particularly notable for its unique structural and functional characteristics, it not only possesses the ability to cleave double-stranded DNA with highly specific sequences (*cis*-cleavage activity) [3] but also cleaves surrounding single-stranded DNA indiscriminately (*trans*-cleavage activity) [4]. Furthermore, beyond its gene editing capability, the CRISPR/Cas12a system has been employed as a powerful signal amplification technique and extensively utilized for detecting a variety of biomarkers, such as DNA [5], RNA [6], small bio-molecules [7] and even living cells [8] with desirable detection sensitivity and detection time. In recent years, researchers further incorporated CRISPR/Cas12a system with some classical nucleic acid amplification techniques such as hybridization chain reaction (HCR), polymerase exponential reaction (PER), and rolling circle amplification (RCA), to accomplish the sensitive detection of trace amounts of biomarkers [9–11]. For example, Long et al. employed a collaborative CRISPR/Cas system, using isothermal PER as a bridge to achieve the sensitive detection of circular RNA, a potential biomarker for acute myocardial infarction (AMI) [12]. In addition, Zhu et al. combined RCA and CRISPR/Cas12a to construct a sensitive one-point "OPERATOR" to achieve rapid detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [13]. In addition, various DNA nanomatrix-CRISPR/Cas systems are also integrated with functionalized inorganic nanomaterials with excellent conductivity, such as *C*–MoS_2, AuNPs/GDY, AuNPs/C–MoS_2, etc., to construct biofuel cells, which not only enables the sensitive detection of biomarkers, but also effectively expands the application of CRISPR/Cas systems [14–17]. Especially using smartphone as signal output device provides a reliable way to

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https://doi.org/10.1016/j.talanta.2025.127730
Received 13 November 2024; Received in revised form 31 January 2025; Accepted 10 February 2025
Available online 12 February 2025
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construct portable detection methods and greatly meets the requirement for remote and real-time on-site monitoring applications [17]. Meanwhile, the dual-mode and multifunctional analysis techniques used in some of these methods also greatly reduce the false positives caused by single-mode detection and improve the accuracy of the methods [16,17]. Despite these advancements, the problem of signal leakage commonly exists in these methods, in which some non-activator nucleic acids are capable of activating Cas effectors to cleave signal reporter molecules (termed "off-target effect") [18–20]. To address this, the Lee group applied chimeric DNA-RNA guides, partially substituting the crRNA with DNA to enhance the binding efficiency between crRNA and activator, and thus reducing the non-specific activation [21]. However, this method is limited in detection sensitivity and complex sample applications. Based on this, a CRISPR/Cas12a system-based detection method with improved detection sensitivity and reduced background signal by avoiding signal leakage is still needed.

Circular RNA (circRNA or ^{C}RNA) molecules are a class of covalent-bonds closed non-coding RNA ring structure. As a new type of RNA molecule, ^{C}RNA exists in large numbers in the eukaryotic transcriptome and is involved in some important transcriptional processes [22–25]. ^{C}RNAs are different from traditional linear RNAs (^{L}RNAs), they have the potential ability to resist RNase degradation and exhibit higher stability than ^{L}RNA, thus greatly prolonging their half-life, so as to achieve the purpose of low dose and long lifespan [26,27]. Based on this, the study of ^{C}RNAs has attracted more and more attention. Recently, the Liu group replaced the traditional linear CRISPR RNA (^{L}crRNA) with ^{C}crRNA in CRISPR/Cas12a-based sensing systems [25], which significantly reduces the background signal. It is the unique topology structure of ^{C}crRNA that masks its normal function, preventing the Cas12a protein exert its role and thus diminishes the background signals greatly. Furthermore, the enhanced stability of ^{C}crRNA contributes to more consistent and reliable detection outcomes, as it is less prone to degradation in complex biological samples.

The antibodies are Y-shaped glycoproteins produced by the immune system [28]. Each antibody comprises a pair of heavy/light chains, forming a variable region that specifically binds to antigens such as viruses, bacteria, and other foreign substances, thereby initiating an immune response [29]. Monitoring antibody levels is crucial for diagnosing diseases, evaluating vaccine efficacy, and understanding immune mechanisms. Based on this, we herein construct a novel CRISPR/Cas12a sensing system with ^{C}crRNA for amplified fluorescent detection of Anti-Dig antibodies in human serum, using the ^{C}crRNA topological structure to effectively suppress the normal function of ^{L}crRNAs, thus reducing the background signal in the absence of target antibodies. In the method, target Anti-Dig antibodies facilitate the assembly of the complete Mg^{2+}-dependent MNAzyme [30] to restore its hydrolysis activity to recognize and cleave the ^{C}crRNA to form ^{L}crRNA, which activates the *trans*-cleavage activity of CRISPR/Cas12a system for further cyclically cleaving large amounts of fluorescent reporter substrate and thus generates a substantially amplified fluorescent signal for the ultra-sensitive detection of Anti-Dig antibodies. In addition, this method combines the quadratic amplification techniques of the CRISPR/Cas12a system [31] and Mg^{2+}-dependent MNAzyme [32], while avoiding the employment of some time-consuming signal amplification techniques such as HCR, RCA, and CHA. Thus, it has great potential to be developed into a simple, sensitive, and reliable biosensing method and applied in clinical diagnostics and biomedical research.

2. Experimental part

2.1. Reagents and materials

Tris-Hydrochloride (Tris-HCl), urea, and $MgCl_2$ were sourced from Sinopharm Chemical Reagent Co., (Shanghai, China). Sheep polyclonal anti-digoxigenin (Anti-Dig), streptavidin (STV), mouse monoclonal anti-2,4-dinitrophenol (Anti-DNP) and mouse monoclonal anti-human immunodeficiency virus (Anti-HIV) were acquired from Sigma-Aldrich Co., Ltd. (Shanghai, China). ELISA detection kit supplied by Jining Bio (Shanghai, China). EnGen® Lba Cas12a, RNase inhibitor, DNase II, T4 RNA Ligase 2 (T4RL2), and RNase R were obtained from New England Biolabs (Beijing, China). All the HPLC-purified oligonucleotide sequences listed below were synthesized by Sangon Biotech Co., Ltd. (Shanghai China) (Table 1).

In the above partzyme A and partzyme B sequences, the **bold** part represents the complementary hybridization region of partzyme A and partzyme B stem, the *italic* part represents the 10–23 DNAzymes' active center, and the underlined region represents the complementary hybridization region with ^{C}crRNA.

2.2. Fluorescence measurements

All fluorescence spectra, excited at 494 nm, were captured using an RF-6000 fluorescence spectrophotometer (Shimadzu, Tokyo, Japan) at 25 °C. The scanning wavelength range is 500–625 nm, and the slit width is set at 5 nm.

2.3. Preparation of the ^{C}crRNA

The solution containing 5′-phosphorylated padlock RNA and linker DNA at a concentration ratio of 1:1.2 was held at 90 °C for 2 min and gradually cooled down to 25 °C within 2 h to form the ^{C}crRNA with a nick. Then the T4RL2 (100 U) was added for 2 h to seal the nick at 37 °C, followed by eliminating the linker DNA and unsealed linear padlock RNA by using the DNase I (50 U) and RNase R (20 U). The formed ^{C}crRNA was spectrally quantified.

Table 1
The used oligonucleotide sequences.

Name	Sequence (5′′→3′)
Padlock RNA	(PO_4)-AUGAUCGUUACGCUAACUAUGAUAAUUUCUACUAAGUGUAG
Linker DNA	AACGATCATCTACACTTA
Activator DNA	TCATAGTTAGCGTAACGATC
Reporter DNA	FAM-TTATTT-DABCYL
Partzyme A_Stem 4	(Dig)-GATACATTAGGCAGA**CGAG***ACAACGA*CATAGTTA
Partzyme A_Stem 6	(Dig)-GATACATTAGGCAGA**CGAGCT***ACAACGA*CATAGTTA
Partzyme A_Stem 8	(Dig)-GATACATTAGGCAGA**CGAGCTAA***ACAACGA*CATAGTTA
Partzyme A_Stem 10	(Dig)-GATACATTAGGCAGA**CGAGCTAAAA***ACAACGA*CATAGTTA
Partzyme A_Stem 12	(Dig)-GATACATTAGGCAGA**CGAGCTAAAAAA***ACAACGA*CATAGTTA
Partzyme B_Stem 4	TAGTAGAAATTA*GGCTAGCT***CTCG**AGACGGATTACATAG-(Dig)
Partzyme B_Stem 6	TAGTAGAAATTA*GGCTAGCT***AGCTCG**AGACGGATTACATAG-(Dig)
Partzyme B_Stem 8	TAGTAGAAATTA*GGCTAGCT***TTAGCTCG**AGACGGATTACATAG-(Dig)
Partzyme B_Stem 10	TAGTAGAAATTA*GGCTAGCT***TTTTAGCTCG**AGACGGATTACATAG-(Dig)
Partzyme B_Stem 12	TAGTAGAAATTA*GGCTAGCT***TTTTTTAGCTCG**AGACGGATTACATAG-(Dig)

2.4. Denaturing polyacrylamide gel electrophoresis (dPAGE) and non-denaturing PAGE

In the dPAGE experiment, the prepared 10 % denaturing gel was initially subjected to a pre-run for 30 min at 220 V in 1 × Tris-borate-EDTA (TBE) buffer. Subsequently, each sample was loaded into the gel and electrophoresed at 120 V for 60 min. Afterward, the gel was imaged after 30 min of staining with Gel-red dye.

For PAGE experiment, a similar procedure was performed. The 10 % native gel was prepared, and the gel with loading samples was run in 1 × TBE buffer at 120 V for 60 min and stained using the diluted Gel-red dye solution.

2.5. Anti-dig antibody detection

The components of partzyme A (100 nM), partzyme B (100 nM), and ^{C}crRNA (150 nM) were firstly mixed in Tris-buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl,10 mM $MgCl_2$). Then target Anti-Dig antibodies with varied concentrations were added and incubated at 37 °C for 30 min. After that, the Cas12a (100 nM), activator DNA (150 nM), and reporter DNA (200 nM) were added and incubated in 1 × NEBuffer r2.1 for another 30 min. The final reaction solution was diluted to 200 μL for measuring the fluorescence intensity.

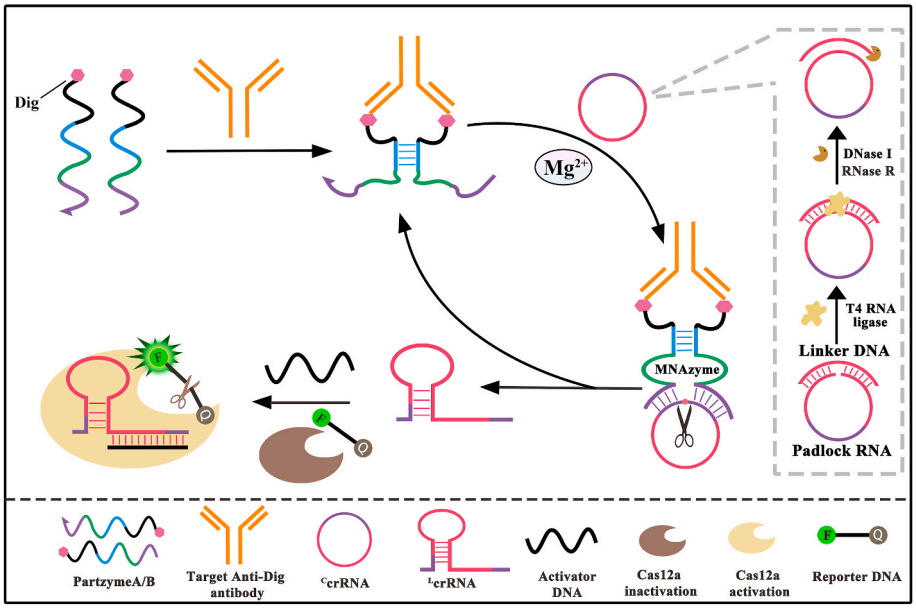
3. Results and discussion

3.1. Detection mechanism of the sensing system

The principle of our proposed low-background CRISPR/Cas12a sensing system with ^{C}crRNA for amplified fluorescent sensing of Anti-Dig antibodies is illustrated in Scheme 1. In this system, two key elements are included, a ^{C}crRNA and a pair of Mg^{2+}-DNAzymes splits (Partzyme A and Partzyme B). The ^{C}crRNA in a topology structure was designed to suppress the function of the ^{L}crRNA, making them unable to regulate the *trans*-cleavage activity of the Cas12a system, thereby diminishing the background signal without target antibodies. Partzyme A and partzyme B are labeled Dig at their 3′ and 5′ end, respectively, each of them contains an arm domain complementary to ^{C}crRNA (purple). In the absence of the target, Partzyme A and Partzyme B are inactive and cannot assemble into an active Mg^{2+}-dependent MNAzyme. However, when target Anti-Dig antibodies are present, they can simultaneously bind with the Dig-labeled partzyme A and partzyme B and make them close to each other to form a complete MNAzyme with recovered enzyme activity. In this case, the two arms of this active MNAzyme can further hybridize with ^{C}crRNA and cleave specific sites (A-U site) of ^{C}crRNA to form ^{L}crRNA with a cofactor of Mg^{2+}. Subsequently, the ^{L}crRNA can bind with Cas12a protein and activator DNA to form the active CRISPR/Cas12a system, which restores the *trans*-cleavage activity of CRISPR/Cas12a and repeatedly cleaves the reporter DNA to produce an amplified fluorescence signal, accomplishing the sensitive detection of Anti-Dig antibodies with low background signal.

3.2. Feasibility of the proposed sensing system

The preparation process of ^{C}crRNA was first characterized using the dPAGE (10 %) experiment and the result is depicted in Fig. 1A. The padlock RNA and linker DNA show two distinct bright bands in lane 1 and lane 2, respectively. Lane 3 contains the mixture of padlock RNA and linker DNA. After adding T4RL2 into the mixture of the padlock RNA and linker DNA, a new bright band was produced in lane 4, which represents the successful formation of the ^{C}crRNA. After further incubating of the RNase R and DNase I, we can see that the upstream lagging bands and the band for the linker DNA both disappear, while only the band corresponding to the ^{C}crRNA still existed (Lane 5), strongly verifying the successful preparation of the ^{C}crRNA. Meanwhile, a native PAGE experiment was performed for the characterization of the target Anti-Dig antibodies-mediated assembly of MNAzyme and the cleavage of ^{C}crRNA. As shown in Fig. 1B, partzyme A, partzyme B, and the synthesized ^{C}crRNA show three bright bands in lanes 1, 2, and 3, respectively. There is an obvious (near the notch) bright band produced in lane 4 after adding target Anti-Dig antibodies while it doesn't exist in the absence of the target, which demonstrated that target antibodies successfully bound with the components of partzyme A and partzyme B and formed the complete MNAzyme structure. After further adding the ^{C}crRNA into above group, the band of ^{C}crRNA dissipates while that for the ^{L}crRNA appears (vs. lane 7), indicating that target-mediated MNAzyme recovered its enzyme activity with the cofactor of Mg^{2+} and hydrolyzed the RNA site in the ^{C}crRNA structure to form the ^{L}crRNA. Additionally, the feasibility of this sensing system was also assessed using fluorescence spectroscopy experiments, with the findings depicted in Fig. 1C. A low fluorescence signal value at 520 nm is recorded (curve a) when the reaction solution only contains the reporter DNA, that



Scheme 1. The mechanism of antibodies detection using the CRISPR/Cas12a sensing system with ^{C}crRNA.

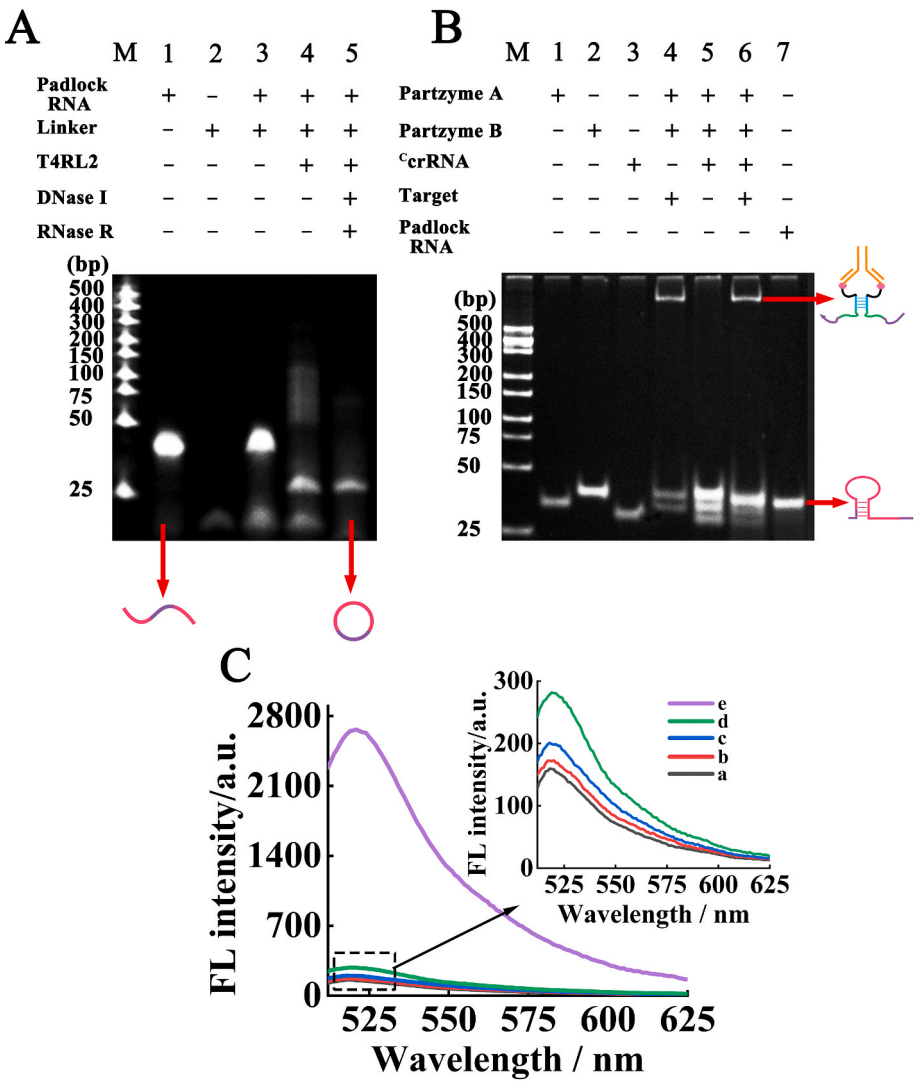


Fig. 1. (A) The dPAGE characterization of the formation of ^{C}crRNA. (B) Native PAGE characterization of target-mediated MNAzymes for the cleavage of ^{C}crRNA. The concentrations of Anti-Dig antibodies, padlock RNA, linker DNA, partzyme A, partzyme B and ^{C}crRNA were 200 nM, 1 μM, 1 μM, 1 μM, 1 μM and 1 μM, respectively, the amount of T4RL2, DNase I and RNase R were 100 U, 50 U and 20 U, respectively. (C) Fluorescence spectra associated with the various reaction solutions: (a) reporter DNA; (b) padlock RNA + Cas12a + activator DNA + reporter DNA; (c) ^{C}crRNA + Cas12a + activator DNA + reporter DNA; (d) ^{C}crRNA + partzyme A + partzyme B + Cas12a + activator DNA + reporter DNA; (e) partzyme A + partzyme B + Anti-Dig antibodies + ^{C}crRNA + Cas12a + activator DNA + reporter DNA. The concentrations of padlock RNA, partzyme A, partzyme B, Anti-Dig antibodies, ^{C}crRNA, Cas12a, activator DNA and reporter DNA were 225 nM, 100 nM, 100 nM, 10 nM, 225 nM, 150 nM, 225 nM and 200 nM respectively. The reaction was carried out at 37 °C for 60 min.

ascribed to the effective fluorescence energy resonance from FAM to DABYCL. The mixture of the padlock RNA, Cas12a protein, activator DNA, and reporter DNA also shows a similar low fluorescence signal (curve b *vs.* curve a), that's because the sequence that binds to the Cas12a protein was split into the 3′ and 5′ ends of the padlock RNA. In this case, the padlock RNA cannot activate the *trans*-cleavage activity of the CRISPR/Cas12a system, and thus no fluorescent signal can be obtained. When the padlock RNA is further replaced with ^{C}crRNA, we found that the fluorescence signal also no change (curve c *vs.* curve b), indicating that the ^{C}crRNA in a topology structure is also unable to activate the *trans*-cleavage activity of CRISPR/Cas12a system. Importantly, when the target Anti-Dig antibodies, partzyme A and partzyme B were further added into the above solution, a significantly enhanced fluorescence signal at 520 nm (curve e *vs.* curve c) is recorded, which verified that target antibodies-mediated MNAzyme is successfully assembled to hydrolyze the ^{C}crRNA into ^{L}crRNA, resulting in the activation of the *trans*-cleavage activity of CRISPR/Cas12a system to digest reporter DNA to produce amplified fluorescent signal. However, in the absence of target antibodies, the fluorescence signal value is reduced to that of the background signal (curve d *vs.* curve a), indicating that the MNAzyme and CRISPR/Cas12a system are both unformed.

3.3. *Optimization of sensing conditions*

The number of complementary base pairs between partzyme A and partzyme B as a key factor is seriously affected the performance of our proposed sensing system, if it is too long, the active MNAzyme can be formed without target Anti-Dig antibodies, and thus a high background signal will be formed; if it is too short, the active MNAzyme is hardly to be assembled even with target antibodies. To achieve this objective, we engineered various lengths of complementary base pairs (from 4-bp to 12-bp with an increment of 2-bp, blue part) between partzyme A and partzyme B (Fig. 2A). As the results shown in Fig. 2B, the value of F and F_0 (F and F_0 denote the fluorescence signal values of FAM with/without target, respectively) are both enhanced with the increase of complementary base pairs, while the F almost approaches saturation after 8-bp. After calculating, we found that the ratio of F/F_0 gets maximum at 8-bp, thus 8-bp was chosen as the optimal complementary base pairs between

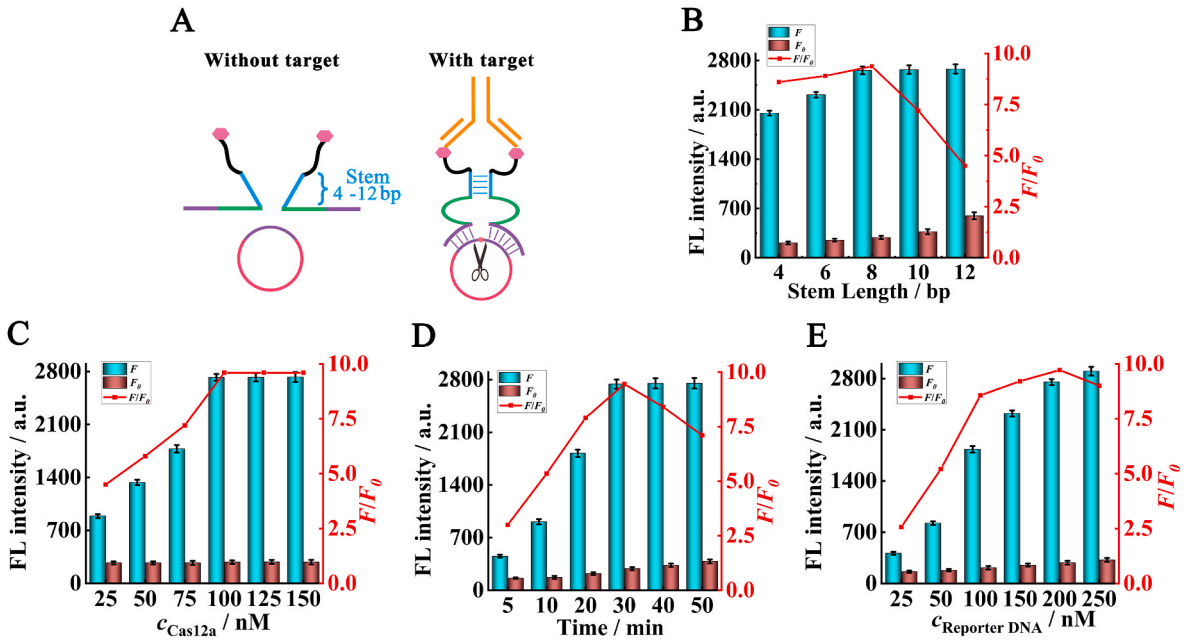


Fig. 2. (A) Design of partzyme A and partzyme B with different complementary stem lengths (ranging from 4- to 12-bp). (B) Plot showing the fluorescent value and F/F_0 of different complementary stem lengths. (C) The concentration optimization of the Cas12a protein. (D) The time optimization of the CRISPR/Cas12a system for degrading reporter DNA. (E) The concentration optimization of the reporter DNA.

partzyme A and partzyme B and used the following. In addition, the amount of Cas12a protein was also investigated. As can be seen from Fig. 2C, as the amount of Cas12a protein increases from 25 nM to 150 nM, the ratio of F/F_0 exhibits a continuous increase, achieving its peak value at a concentration of 100 nM, thus 100 nM was defined as the optimal amount of Cas12a. Furthermore, the reaction time for the active CRISPR/Cas12a system to degrade the reporter DNA was also optimized. Fig. 2D shows that the value of F/F_0 reaches a peak at 30 min, indicating that the CRISPR/Cas12a system can completely degrade reporter DNA within 30 min. Finally, the concentration of reporter DNA was optimized and a similar result was obtained in Fig. 2E. It shows that both F and F_0 increase as the reporter DNA concentration increased from 25 nM to 250 nM, and the maximum value of F/F_0 is obtained at 200 nM, which is defined as the optimal concentration of reporter DNA.

3.4. Analytical performance

To assess the analytical capability of the developed CRISPR/Cas12a system with ^{C}crRNA for target antibodies, we monitored fluorescence responses to Anti-Dig antibodies in a range of different concentrations under established optimal experimental conditions. The results are depicted in Fig. 3A, as the concentration of target antibodies increases from 0 nM to 50 nM, there is a corresponding elevation of the fluorescence response. By correlating the target antibodies concentration with its fluorescence emission at 520 nm, a linear regression equation of $F = 188.02c + 670.6253$ (F and c represent the fluorescence emission at 520 nm and concentration of target antibodies, respectively) was obtained, demonstrating a good linear relationship in the concentration range of 25 pM–50 nM, with a correlation coefficient (R^2) of 0.9976 (Fig. 3B). Utilizing the 3σ rule, 15 pM was determined as the detection limit of the method. Compared to many CRISPR/Cas12a-based single signal amplification sensing methods in Table 2, such a wider linear detection range and a lower detection limit are basically owing to the two amplification steps involved in our assay protocol. Additionally, the reaction times for each method have been included in our new Table 2, the rapid response time of MNAzyme to RNA substrate (almost 5 min) and active CRISPR/Cas12a system to ssDNA reporters (30 min) enable our method to achieve sensitive detection of target Anti-Dig antibodies within 60 min, which is superior to some classical signal amplification methods (over 2 h), such as HCR and RCA.

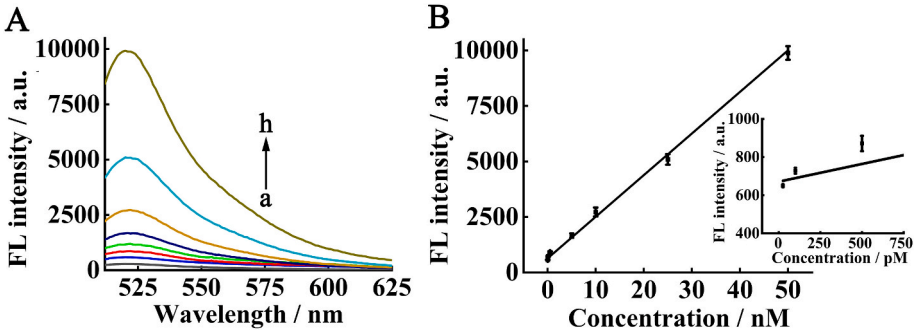


Fig. 3. (A) Fluorescence spectra to different concentration of Anti-Dig antibodies from 0 to 50 nM (0, 0.025, 0.1, 0.5, 5, 10, 25, 50 nM). (B) Corresponding calibration curves of fluorescence emission intensity at 520 nm to concentration of Anti-Dig antibodies from 25 pM to 50 nM. Error bars, mean ± SD based on three independent measurements.

Table 2
Different fluorescent methods for detecting Anti-Dig antibody.

Detection mechanism	Linear range	Detection limit	Detection time	Reference
Programmable catalytic molecular nanomachine	1 nM–250 nM	0.85 nM	4 h	[33]
Sensitive CRISPR/Cas12a assisted immunoassay	63 pM-16 nM	63 pM	60 min	[34]
CRISPR/Cas12a-powered immunosorbent assay	0.125 nM–125 nM	0.125 nM	60 min	[35]
A novel switchable DNA tweezers	100 pM-100 nM	88 pM	50 min	[36]
A fluorescent probe containing a coiled-coil peptide pair E4/K4	0.64 nM–1.2 nM	0.19 nM	30 min	[37]
CRISPR/Cas12a sensing system coupled with ^{C}crRNAs and MNAzyme	25 pM-50 nM	15 pM	60 min	This work

3.5. Selectivity

Another key parameter used to evaluate the analytical performance of Anti-Dig antibodies is selectivity. To this purpose, three interfering proteins of Anti-DNP, STV, and Anti-HIV antibodies were selected as the control groups and used for fluorescence monitoring. As shown in Fig. 4, the corresponding fluorescence response values from the three control groups are still at a very low level even their concentrations (100 nM) are 10 times higher than that of the target antibodies (10 nM). However, target Anti-Dig antibodies (10 nM) produce a significantly increased fluorescence responsive value. Importantly, the fluorescence signal produced by the mixture of the three interfering antibodies and the target antibody was comparable to that produced by the target antibody alone. These results confirmed that only target antibodies can bind with partzyme A and partzyme B to form the complete MNAzyme structure with recovered enzyme activity, which further activates the *trans*-cleavage activity of the CRISPR/Cas12a system to generate an essentially enhanced fluorescent signal. Moreover, even in complex environments where multiple interfering proteins co-exist, our approach is also highly selective for the target antibody without interference from other components.

3.6. Simulated sample analysis

To further demonstrate the potential applicability of our strategy for target Anti-Dig antibodies, a standard recovery experiment of Anti-Dig antibodies at different concentrations in diluted human serum was performed. The recoveries obtained in Table 3 ranged from 96.16 % to 103.08 %, which is an ideal narrow recovery range, indicating that our strategy has great potential in the analysis of Anti-Dig antibodies in simulated biological samples. In addition, a mature enzyme-linked immunosorbent assay (ELISA) method was employed to test various concentrations of target antibodies, the corresponding detection principle and analysis steps are shown in Fig. S1. From the results listed in Table 3, the recoveries obtained by ELISA are ranged from 99.12 % to 101.64 %, and the two results from our method and ELISA are in close agreement with each other, which well verifies the accurate of our method.

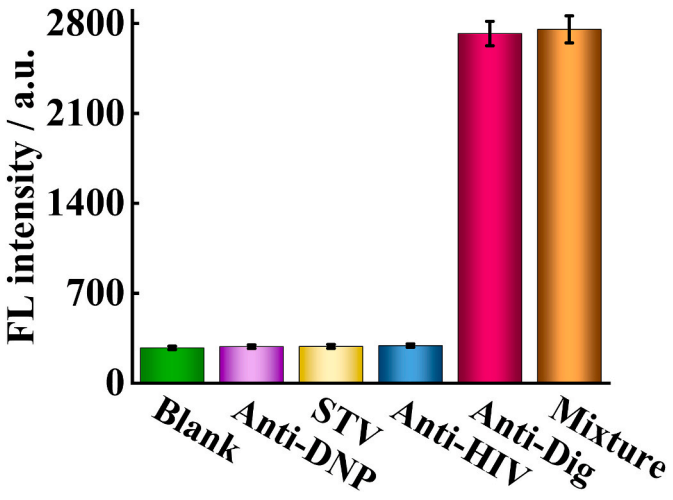


Fig. 4. Studies based on the selectivity of target antibody against other antibodies: Anti-DNP, STV and Anti-HIV antibodies (100 nM). Error bars, mean ± SD based on three independent measurements.

4. Conclusion

In summary, a low-background CRISPR/Cas12a sensing system with ^{C}crRNAs in a topology structure is constructed for monitoring Anti-Dig antibodies in diluted human serum based on the quadratic signal amplifications of MNAzyme and CRISPR/Cas12a. The ^{C}crRNA used in this work greatly suppresses the function of the ^{L}crRNAs, making them unable to regulate the *trans*-cleavage activity of the Cas12a system and reducing the background signal in the absence of target antibodies. The detection system achieves the ultrasensitive fluorescent detection of Anti-Dig antibodies down to 15 pM within 60 min, good selectivity toward other interfering proteins, as well as successful application in diluted serum samples. In addition, the quadratic signal amplification strategy in this method can be easily applied to monitor other antibodies with dual recognition sites or proteins with double recognition aptamers, such as Anti-DNP, Anti-HIV, Anti-cTnI (cardiac troponin I antibody), thrombin, etc. Currently, the CRISPR/Cas12a-based signal amplification technologies are mostly applied for developing fluorescent or electrochemiluminescent biosensing methods, which require professional instruments and operators. Future work may focus on exploring portable sensing devices by combining simple and easily accessible signal transmission tools (e.g., mobile phones) for highly sensitive and accurate detection of the biomarkers that can be envisioned in the near future.

CRediT authorship contribution statement

Qian Xiang: Writing – original draft, Methodology, Formal analysis, Data curation. **Fang Guo:** Methodology, Data curation. **Daxiu Li:** Formal analysis. **Fengfeng Xu:** Supervision, Formal analysis, Conceptualization. **Wenjiao Zhou:** Writing – review & editing, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

Table 3
Standard recovery test for Anti-Dig antibodies in 10-fold diluted serum samples (n = 6).

Sample	Added (nM)	This work		ELISA	
		Found (nM, mean ± SD)	Recovery (%)	Found (nM, mean ± SD)	Recovery (%)
1	0	Not found	–	Not found	–
2	1	1.01 ± 0.09	101.42	1.01 ± 0.02	101.14
3	2	2.06 ± 0.14	101.25	1.98 ± 0.08	99.73
4	5	5.15 ± 0.18	103.08	5.08 ± 0.12	101.64
5	10	9.91 ± 0.43	98.77	9.91 ± 0.24	99.12
6	20	19.23 ± 1.20	96.16	–	–

the work reported in this paper.

Acknowledgments

This work was supported by National Natural Science Foundation of China (No. 22004010), the Science and Technology Research Program of Chongqing Municipal Education Commission (KJQN20230111) and the Chongqing Science and Technology Commission of China (2023NSCQ-MSX1563).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2025.127730.

Data availability

Data will be made available on request.

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