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Dynamic bidirectional diffusion-controlled multi-enzyme system for one-pot viral detection

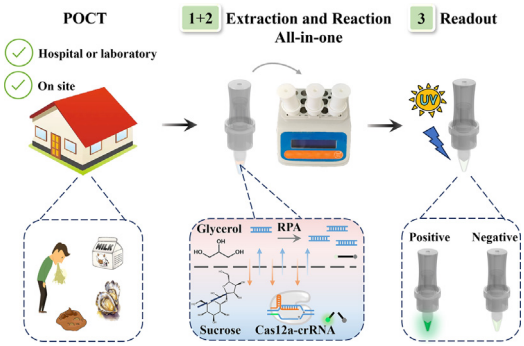
Jing Zhang [a], Xuening Shi [a], Yukun Ding [a], Jingying Zheng [a], Hang Li [a], Hongran Zhao [b], Tong Zhang [b], Qiuyu Xing [a], Chao Zhao [a,*], Shuo Yao [a,*], Juan Wang [a,*]

[a] State Key Laboratory for Diagnosis and Treatment of Severe Zoonotic Infectious Diseases, Key Laboratory for Zoonosis Research of the Ministry of Education, School of Public Health, Jilin University, Changchun 130021, China
[b] College of Electronic Science and Engineering, Jilin University, Changchun 130012, China

HIGHLIGHTS

- A dynamic bidirectional diffusion-controlled multi-enzyme system has been established.
- Diffusion-controlled strategy addressing the incompatibility between RPA and CRISPR.
- The entire detection process is completed in less than 35 min achieving a single copy level.
- The all-in-one device integrates the RPA and CRISPR reaction in a one-pot reducing aerosol contamination.
- The all-in-one device is ultra-simple without extra operation and instruments.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Integrating isothermal amplification with CRISPR-based detection in a single reaction vessel holds significant promise for rapid and sensitive point-of-care virus diagnostics. However, conventional one-pot methods often suffer from mutual inhibition between amplification and CRISPR-Cas reactions, which compromises detection performance and limits their practical application.
Objectives: This study aims to overcome the inhibitory interactions between isothermal amplification and CRISPR-based detection by developing an integrated reaction system that enables efficient multi-enzyme coordination within a single tube.
Methods: We designed a dynamic bidirectional diffusion-controlled RPA/CRISPR-Cas12a multi-enzyme system based on a dual-phase separation strategy. In this system, glycerol was used to modulate viscosity and accelerate the RPA reaction, while sucrose was introduced to create a density gradient that enables spatial separation. This configuration effectively coordinates the activities of multiple enzymes within one reaction vessel. Furthermore, a 3D-printed nucleic acid extraction device was integrated to simplify sample preparation and enhance overall detection efficiency.
Results: The developed system achieved single-copy sensitivity and completed detection within 30 min, exhibiting over 100-fold higher sensitivity than conventional one-pot assays. The method was further validated by detecting Norovirus in both clinical and food samples, confirming its robustness and accuracy.

* Corresponding authors.
E-mail addresses: czhao0529@jlu.edu.cn (C. Zhao), yaoshuo@jlu.edu.cn (S. Yao), jwang0723@jlu.edu.cn (J. Wang).

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Conclusion: The proposed dual-phase RPA/CRISPR-Cas12a system provides a simple, rapid, and highly sensitive platform for nucleic acid detection. Its operational simplicity, compatibility with low-resource settings, and potential for home-based diagnostics highlight its strong applicability for decentralized virus detection.

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1. Introduction

In recent years, the world has witnessed a sharp rise in major viral outbreaks, such as SARS-CoV-2 and monkeypox. Among them, SARS-CoV-2 has claimed an estimated 16 million lives globally within just two years, while also shaving 1.6 years off the average life expectancy worldwide [1]. In this context, rapid and efficient diagnostic tools are essential to contain disease transmission. Foods and packaging can become contaminated during production and distribution, serving as fomites for pathogen transmission via infected handlers, contaminated water, or surfaces [2,3]. Viral RNA, like SARS-CoV-2, has been found on food and packaging, especially in cold-chain settings [4,5]. Nucleic acid detection methods are a powerful tool in molecular diagnostics, directly identifying DNA or RNA to minimize interference from food components. They offer high sensitivity by exponentially amplifying target sequences, allowing for the detection of very low nucleic acid copy numbers [6]. These advantages make them particularly suitable for food safety and disease prevention and control, allowing quick and sensitive pathogen identification to prevent outbreaks and safeguard public health [7,8]. While the most frequently applied quantitative PCR (qPCR) remains the gold standard in the detection of low-abundance nucleic acids [9–11], its reliance on centralized laboratories, extended turnaround time (4–24 h), and high operational costs limit its applicability in timely outbreak response. These challenges underscore the urgent need for rapid, sensitive, and user-friendly point-of-care testing (POCT) systems for decentralized and field-based diagnostics.

POCT technologies, including antigen assays and isothermal amplification methods such as RPA, LAMP, and RCA [12], enable detection within 10–30 min. However, their clinical utility is hindered by suboptimal sensitivity and high false-negative rates. Clustered regularly interspaced short palindromic repeats (CRISPR) technology, particularly the CRISPR-Cas12a system, has garnered considerable attention for its simplicity, programmability, and potent *trans*-cleavage activity, which enables robust signal amplification [13]. By coupling CRISPR-Cas12a with isothermal amplification techniques, such as RPA or LAMP, CRISPR-based methods achieves enhanced sensitivity and specificity in molecular diagnostics [14,15], offering a promising solution for decentralized and timely public health surveillance. However, early CRISPR-based assays, including DETECTR and HOLMES, required a two-step workflow involving sequential amplification and CRISPR-Cas12a cleavage [16,17], which increased operational complexity and risk of aerosol contamination. To streamline diagnostics, one-pot CRISPR systems have emerged, combining both reactions within a single vessel [18,19]. Nevertheless, a central challenge in one-pot CRISPR-based detection systems is the incompatibility among enzymes, where uncontrolled Cas12a *trans*-cleavage and mutual interference between components can disrupt amplification and compromise assay performance [20,21]. Although the CRISPR-Cas12a components can be placed on the tube lid or tube with insert strategy [22–24], or blocked by photocleavable modifications [25–27], these strategies typically require an additional centrifugation step or UV irradiation after the completion of the RPA reaction to allow the two reactions to proceed sequentially within a single tube, which are two-step detection process.

Existing strategies to address the incompatibility between RPA and CRISPR-Cas systems in one-pot assays can be broadly categorized into two approaches: spatial separation of reagents and chemical modulation of enzymatic activity through reaction additives [21,28–31]. These approaches improve detection efficiency by either physically isolating enzymatic reactions to prevent interference or by modulating enzyme kinetics to better synchronize amplification and CRISPR activities [28,32]. For example, hydrogels, wax-based or microfluidic chip phase separation have been employed to segregate RPA and CRISPR reagents until the completion of isothermal amplification [33–35]. Among these strategies, hydrogel-confined technology takes advantage of the natural porous network, whose tunable pore size enables effective separation of nucleic acids and proteins [29]. The targeted nucleic acid was amplified in RPA reagents and not affected by the Cas protein. As the RPA reaction progressed, the amplification product diffused and reached the CRISPR system through the hydrogel network and activated the Cas12a trans-cleavage activity. Hydrogel also offers excellent biocompatibility with minimal interference in nucleic acid amplification and enzymatic activity [36]. In addition, hydrogel is easy to prepare [29]. Due to their physical separation characteristics, excellent biocompatibility, and ease of preparation, hydrogels are highly suitable for development of high sensitivity, portability detection methods [29]. However, hydrogel suffers from restricted mass transport, poor mechanical robustness, and challenges in scalable manufacturing. Alternatively, additives such as polyethylene glycol (PEG) or heparin sodiume have been demonstrated to enhance amplification efficiency or delay CRISPR activity [28,37], thus facilitating more coordinated multi-enzyme reactions. In the RPA system, PEG acts as a crowding agent and is known to promote condensate formation through the crowding effect of protein, thereby enhancing amplification efficiency. Meanwhile, heparin sodium inhibits Cas12a's cleavage of trace substrates to enables RPA to perform extensive nucleic acid amplification, and the nucleic acid amplification products reach a certain concentration, Cas12a's cleavage activity is no longer effectively inhibited, thereby realizing one-pot RPA-Cas12a reaction. In light of the above, combining spatial separation with biochemical modulation may present a promising strategy to improve reaction coordination and expand the applicability of one-pot assays for viral POCT.

Here, we present a dynamic bidirectional diffusion-controlled RPA/CRISPR-Cas12a system that integrates spatial and temporal regulation of multi-enzyme activity using two simple chemical additives--glycerol and sucrose. In this system, glycerol increases the local viscosity to enhance the reaction rate of RPA, while the combination of glycerol and sucrose establishes a density gradient that enables phase separation between the amplification and CRISPR-Cas12a components. This coordinated regulation effectively mitigates enzyme interference, thereby addressing the compatibility challenges inherent in one-pot assays. As illustrated in Fig. 1, conventional one-pot detection assay consists of three modules: nuclear acid extraction, RPA/CRISPR reaction, and results readout. While our integrated detection platform realizes the integration of nucleic acid extraction and reaction in one module, simplifying sample pretreatment steps and shortening detection time. Benefiting from this spatial-temporal regulation, the assay

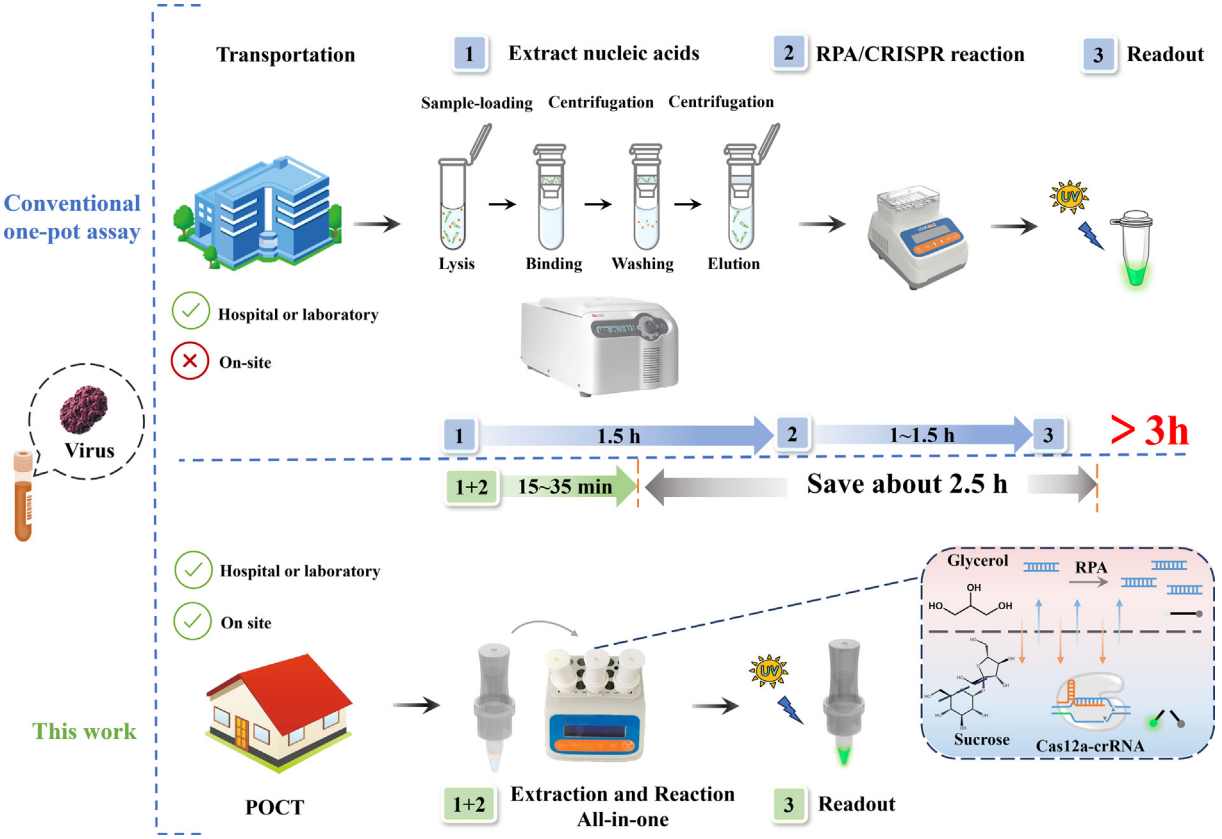


Fig. 1. Flowchart of virus detection based on one-pot dynamic bidirectional diffusion-controlled multi-enzyme. Our work detection process includes one-pot sample nucleic acid extraction, and dynamic bidirectional diffusion-controlled multi-enzyme system. Conventional one-pot assay usually takes more than 3 hours to report results (top), which requires longer detection time. In comparison, our work can provide results about 35 min (bottom), simplifying operation steps and without large instruments.

achieved single-copy sensitivity within 30 minutes and exhibited over 100-fold greater sensitivity than conventional one-pot assay. The platform was further validated by detecting Norovirus in clinical and food samples, demonstrating excellent analytical performance, minimal operational complexity, and strong potential for point-of-care applications, particularly in decentralized or resource-limited settings.

2. Material and methods

2.1. Materials

The TwistAmp Basic RPA Kit was purchased from TwistDx, Inc. (Cambridge, UK). Norovirus GII Real Time RT-PCR Kit (MEIZHENG BIO-TECH, Shandong, China) was purchased for the standard RT-qPCR detection. The plasmid template of Norovirus GII and other oligonucleotide strands were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). All The complete sequences are listed in Supplementary Table S1. The sucrose was purchased from Beijing Chemical Industry Group (Beijing, China), and glycerin was obtained from Sangon Biotech Co., Ltd. (Shanghai, China). NEBuffer™ r2.1 was obtained from New England Biolabs. Reverse transcriptase, RNase inhibitor, random primers and dNTP were purchased Sangon Biotech Co., Ltd. (Shanghai, China). All reagents mentioned were of analytical grade. The School of Public Health at Jilin University provided deionized water. Fluorescence signal was recorded by QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, Massachusetts, USA).

2.2. Characterization of hydrogen bond network structure in sucrose and glycerol

The characterization of hydrogen bond network was conducted by Raman spectrometer. The protocol of the Raman measurements is described in the Supplementary Data.

2.3. Molecular Dynamics Simulation of hydrogen bond

The chemical structure of a sucrose was manually constructed in Discovery Studio Visualizer. A total of 5 sucrose molecules were obtained. Then 171 water molecules were added for each sucrose. Finally, the size of the simulation system was 34*27*34 Å3, containing 2790 atoms. The detail on molecular dynamics simulation is described in Supplementary Data.

2.4. Design of RPA primers and crRNAs

The RPA primers were designed according to the experimental design manual provided by TwistDx, and meeting the following conditions. The RPA primer length is 30–35 nt and the amplicon length is 100–200 bp. The target gene sequences were selected from the conserved sequence region of the GII genotype Norovirus. Additionally, the designed primers are non-overlapping with the crRNA, and the resulting amplicon contains a specific crRNA binding site. After primer design, the primer sequences were compared against nucleic acid sequences in the NCBI database using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to evaluate

their specificity. Specific primers with high amplification efficiency were further screened experimentally. Within the amplified fragments, crRNAs were designed, consisting of a repeat sequence (5′-AAUUUCUACUAAGUGUAGAU-3′) and a target sequence (with base "T" replaced by "U"). The designed crRNA sequences were aligned against available sequences in the NCBI GenBank database using the BLAST program to preliminarily confirm the absence of highly homologous sequences elsewhere.

2.5. The dynamic bidirectional diffusion-controlled (RT-)RPA/CRISPR-Cas12a system

For the dynamic bidirectional diffusion-controlled RPA/CRISPR-Cas12a assay, the RPA reaction was conducted in a 50 μL system. The 35 μL Cas12a reaction was conducted using 150 nM LbCas12a, 200 nM crRNA, 1 × NEBufferT^M 2.1, 5 μM reporter, and 10% sucrose, and the 15 μL RPA reaction contains 500 nmol/L of both forward and reverse primers, 7 mmol/L MgOAc, and 10% glycerin. The 2.5 μL sample (or 2.5 μL RNase/DNase-free water in negative control) was added to each reaction. Fluorescence signals were recorded by a QuantStudio™ 5 Real-Time PCR System. The reaction was carried out at 42℃ for 30 min, and the fluorescence signal was recorded every minute. For the dynamic bidirectional diffusion-controlled RT-RPA/CRISPR-Cas12a assay, the components were the same as those in the RPA-CRISPR/Cas12a assay above, except supplementing 200 U/μL reverse transcriptase, 2 U/μL RNase inhibitor, 50 pmol/μl random primers, 1 mol/μl dNTP.

2.6. Milk and oyster sample preparation

A total of 7 milk samples were spiked with 1.54, 3.07, 30.7, 3.07×10^2, 3.07×10^3, 3.07×10^4, 3.07×10^5 copies/μL of GII Norovirus plasmid. Seven of oyster samples were prepared the same as spiked milk samples.

2.7. Clinical fecal sample preparation

Nine negative and nineteen positive clinical human fecal samples were obtained from Changchun Children's Hospital. All samples were frozen at -80℃ before detection. The research work has been approved by the Ethics Committee of the School of Public Health, Jilin University (No. 2024–07-11). All the collected samples were confirmed for Norovirus by RT-qPCR assay before use. The protocol of the RT-qPCR is described in the Supplementary Data.

2.8. Norovirus nucleic acids extraction device and the detection on all-in-one detection device

The ultrasensitive detection platform of Norovirus in fecal samples was performed using Norovirus detection system. Briefly, the fecal clinical samples were first diluted to 10-fold volume with PBS buffer. 200 μL of the supernatant was added into the four hundred microliters of the lysis buffer (4 M guanidine thiocyanate, 55 mM Tris-HCl, 25 nM EDTA, and 3% Triton X-100, 20 μL proteinase K, 5 μL Carrier RNA). The mixture was incubated at room temperature for 2 min, and dropped into the middle of span column for nucleic acid extraction. Then, the span column was washed two times with wash buffer (10 mM Tris-HCl, 80% ethanol), and 200 μL ethanol. After elution, the span column was transferred onto the tube. Then, the span column was washed with 20 μL RNase/DNase-free water, and inject into dynamic bidirectional diffusion-controlled RPA-CRISPR/Cas12a reaction system. The reaction proceeded at 42℃ for 30 minutes, resulting in an observable fluorescence signal.

2.9. Statistical analysis

Significance testing was conducted using a two-sample t-test, with a significance level set at $P < 0.05$. The results were shown as mean ± standard error. Significance thresholds were set as follows: NS $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results and discussion

3.1. Working principle of the dynamic bidirectional diffusion-controlled multi-enzyme system

To elucidate the working principles of the bidirectional diffusion-controlled RPA/CRISPR-Cas12a multi-enzyme system, we systematically investigated the effects of additive placement, concentration ratios, and reaction temperature on assay performance. Using Norovirus plasmid as a model target, we first examined the effects of additive type and spatial positioning by testing 16 different combinations of glycerol and sucrose across the RPA and CRISPR-Cas12a layers (Fig. 2a and b and Supplementary Fig. S1). Fluorescence signals were highest when positioning the RPA reaction in the upper layer and the CRISPR-Cas12a reaction in the lower layer, compared to other configurations. This is likely because glycerol could enhance RPA kinetics by increasing local viscosity, and sucrose could establish a density gradient that facilitates phase separation and delays premature interaction between amplification and CRISPR cleavage components. To further validate this observation, we further evaluated the effect of sucrose-to-glycerol ratios (ranging from 1:1 to 1:0.1), with sucrose fixed at 10% (w/v) according previous work [28], and the strongest signal was observed at a ratio of 1:0.75 (Fig. 2c). Under this condition, the upper glycerol layer exhibited a density of ~1.024 g/cm^3 and the lower sucrose layer ~1.038 g/cm^3.The density distribution of being smaller at the top and larger at the bottom ensures stable stratification within the single-tube system. Moreover, the RPA reaction rate in the glycerol-containing system was nearly double that in water (Supplementary Fig. S2), highlighting the role of glycerol in accelerating amplification. These findings suggest that glycerol enhances RPA kinetics by increasing local viscosity, while sucrose establishes a density gradient that facilitates phase separation and delays premature interaction between amplification and detection components. Having optimized the additive combination and concentration ratio, we next examined key reaction parameters. The bidirectional diffusion-controlled RPA/CRISPR-Cas12a system exhibited excellent temperature tolerance within the range of 37–45℃ (Fig. 2d and Supplementary Fig. S3). And the fluorescence signal reached a plateau after 30 min and remained stable for at least 60 min, with RSD = 3.62% over 30 minutes at 42℃, indicating reliable temporal performance of the readout (Supplementary Fig. S4). Compared with one-pot reactions relying solely on sucrose or glycerol (about 60 min)[28,32], this system achieved detection in a significantly shorter time. Interestingly, we found placing the reporter of CRISPR-Cas12a system above the glycerol-containing RPA phase yielded the most efficient signal generation (Fig. 2e). It indicates that the amplification product of the target does not merely diffuse downward; there is also an upward diffusion behavior of the cas12a cleavage system. Compared with the conventional one-step assay, this study significantly improved the detection signal and shortened the detection time, which achieved a 39-fold signal enhancement within 30 minutes using 3.07×10^5 copies/μL of GII Norovirus plasmid (Fig. 2f-i).

To further elucidate this behavior, we systematically investigated the interlayer transport of amplification products and CRISPR components (Fig. 3). Double-stranded RPA products generated in the upper glycerol layer diffused downward into the CRISPR layer

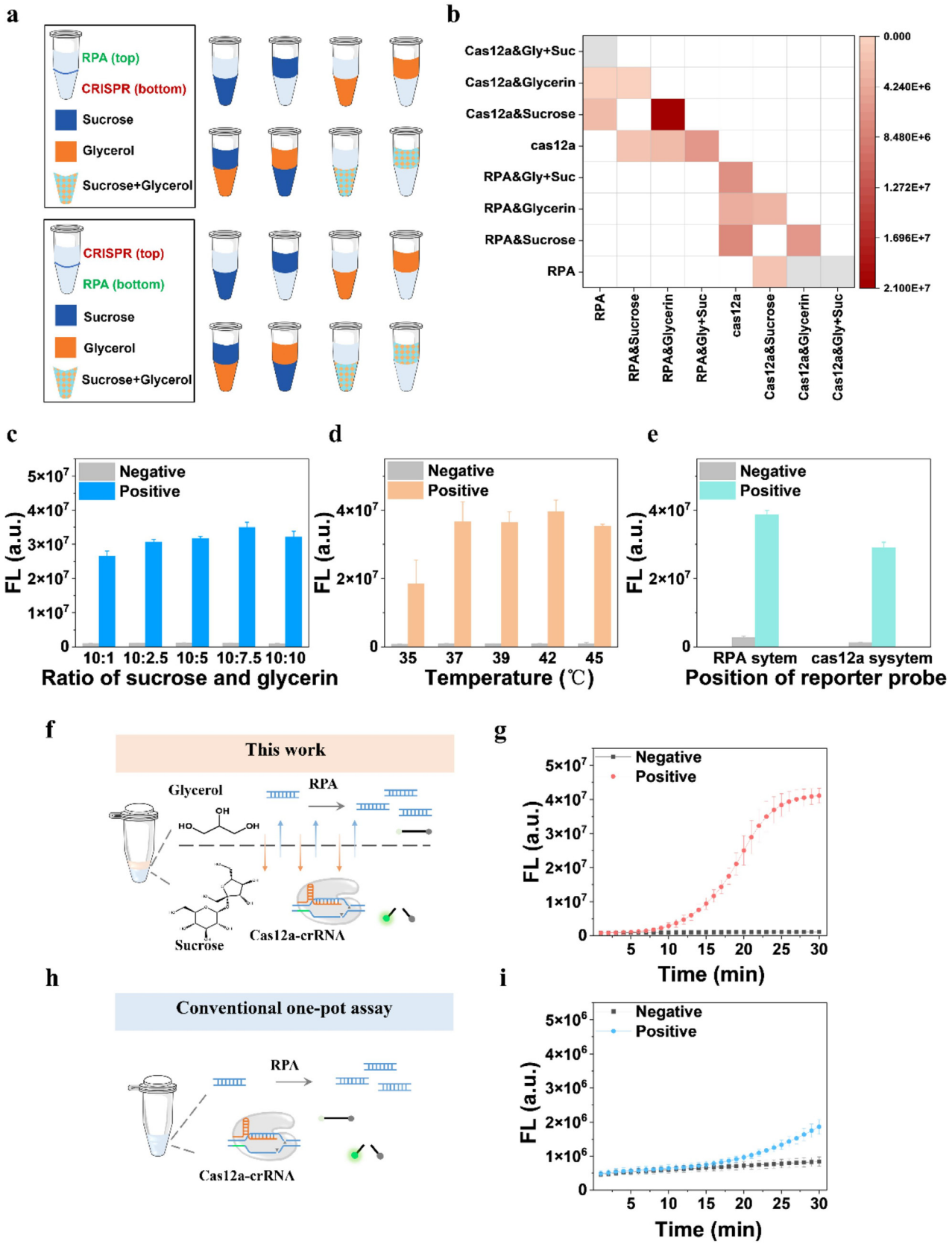


Fig. 2. Establishment of dynamic bidirectional diffusion-controlled multi-enzyme system. a-b) Schematic and fluorescence intensity of different combinations of glycerol and sucrose across the RPA and CRISPR-Cas12a layers. c) Evaluation of the effects of the ratio of sucrose and glycerin. d) Evaluation of the effects of the reaction temperature. e) Evaluation of the effects of the position of fluorescence report probe. f-g) Schematic and the fluorescence curve of dynamic bidirectional diffusion-controlled multi-enzyme system. h-i) Schematic and the fluorescence curve of conventional one-pot RPA/CRISPR-cas12a assay. The detection assay containing either 3.07×10^5 copies/μL of GII Norovirus plasmid (Positive) or no GII Norovirus plasmid (Negative).

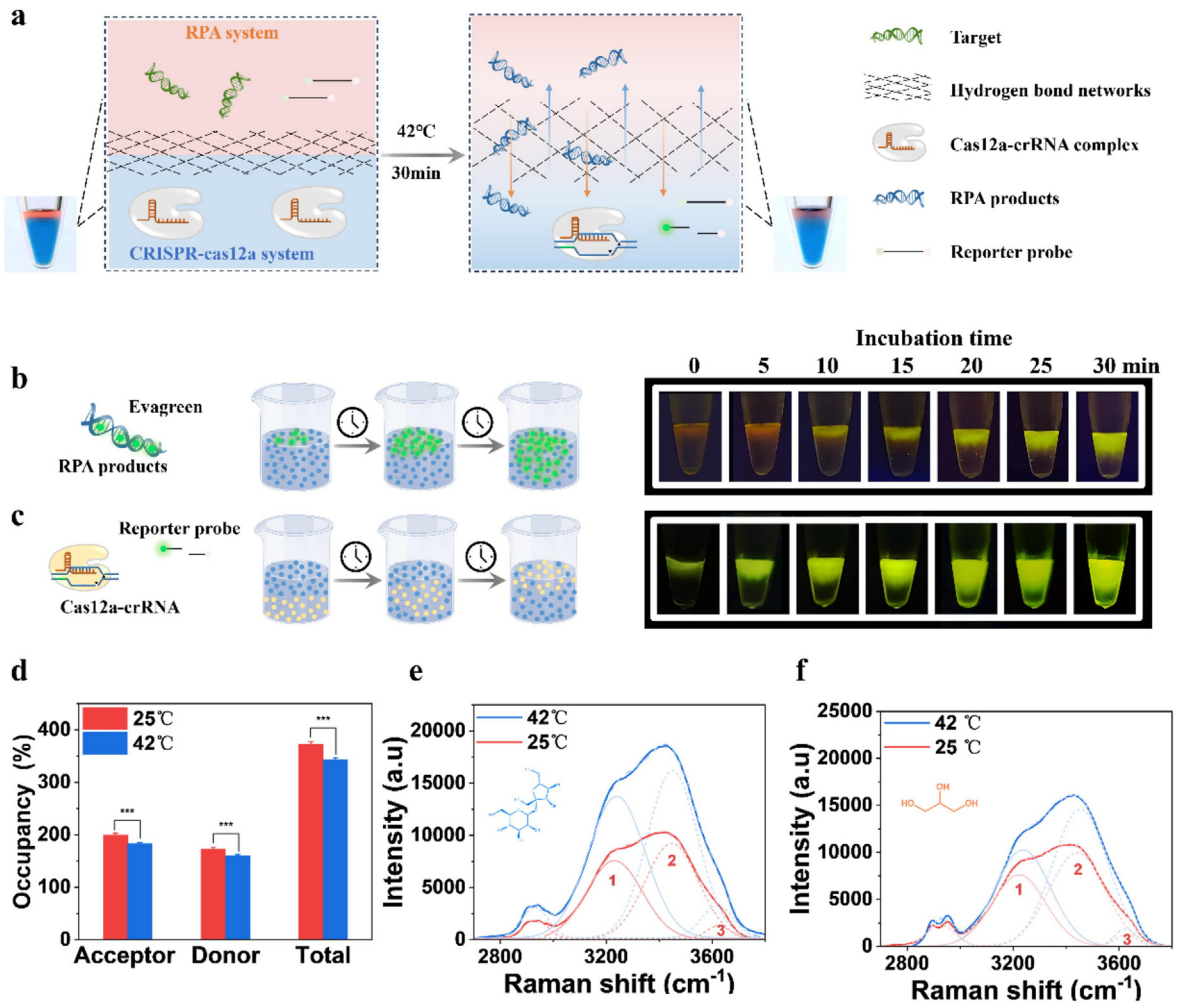


Fig. 3. Dynamic bidirectional diffusion-controlled mechanism of sucrose and glycerin. a) Schematic diagram of dynamic bidirectional diffusion-controlled RPA/CRISPR-Cas12a assay. The mixtures of CRISPR system and sucrose were on the bottom of the tube (with blue dye), and the mixtures of RPA and glycerol were on top (with red dye). b) Schematic diagram and fluorescence image of PRA product dynamically diffusing downwards into CRISPR system. c) Schematic diagram and fluorescent photograph of dynamic upward diffusion of cas12a and crRNA into RPA system. d) Molecular dynamics simulation of hydrogen bond. Hydrogen bond occupancy for donor, acceptor and total of 10% sucrose solutions at 25℃ and 42℃. e) The Raman spectroscopy of 10% sucrose solutions. f) The Raman spectroscopy of 10% glycerol solutions. Hydrogen bonds proton donors (D) or proton acceptors (A), sub-peaks are sequentially defined as S1(DDAA), S2 (DA) and S3 (DDA + free O–H). Dynamic bidirectional diffusion-controlled RPA/CRISPR-Cas12a assay containing either 3.07×10^5 copies/μL of GII Norovirus plasmid (Positive) or no GII Norovirus plasmid (Negative).

to activate Cas12a, while Cas12a protein and associated small components in the lower CRISPR layer were capable of diffusing upward into the RPA layer (Fig. 3b-c). This bidirectional exchange occurs within the density-structured multiphase system and is facilitated by hydrogen-bond network relaxation at elevated temperature, distinguishing it from conventional unidirectional diffusion driven solely by concentration gradients. To visualize the spatial progression of amplification, EvaGreen, a double-stranded DNA-binding dye, was introduced into the glycerol-containing RPA system. As shown in Fig. 3b, within the first 15 min, the fluorescent products remained confined to the upper layer. As the reaction progressed, they gradually diffused into the lower layer. Then, the CRISPR fluorescent reporter probes were placed in the upper layer, while retaining Cas12a and crRNA in the sucrose-containing lower layer. After 5 min, fluorescence signals emerged in the upper RPA layer, indicating that the CRISPR components (Cas12a and crRNA) from the lower layer gradually diffused upward into the amplification zone (Fig. 3c). In addition, at 25℃, the network remained largely stabilized and the one-pot RPA-CRISPR detection failed to reaction (Supplementary Fig. S5). To further quantify this bidirectional diffusion behavior, colored dyes were introduced into the system to visually track the phase interface over time (Supplementary Fig. S6-7). Gray-value image analysis revealed that during the progressive diffusion between the RPA and CRISPR phases, the center position of the diffusion layer remained nearly unchanged (Supplementary Fig. S7), which is characteristic of diffusion behavior described by Fick's second law. To further quantitatively evaluate the bidirectional diffusion process, molecular dynamics (MD) simulations were performed (Supplementary Fig. S8). The simulation results showed that dsDNA molecules located in the upper RPA phase diffused toward the CRISPR phase with a diffusion coefficient of 0.3593×10^{-7} $cm^2\ s^{-1}$, while Cas12a–crRNA complexes in the lower CRISPR phase diffused upward with a diffusion coefficient of 0.1782×10^{-7} $cm^2\ s^{-1}$. For comparison, the diffusion coefficient of nucleic acids in conventional aqueous systems is typically around 1.42×10^{-7} $cm^2\ s^{-1}$ [38,39], whereas proteins generally exhibit diffusion coefficients on the order of $0.61–1 \times 10^{-7}$ $cm^2\ s^{-1}$ [40]. The relatively lower diffusion coefficients observed in the present biphasic system suggest that sucrose and glycerol reduced molec-

ular mobility, which is consistent with the increased viscosity and enhanced intermolecular interactions introduced by these components. These quantitative results demonstrate that both amplification products and Cas12a-crRNA complexes can migrate across the phase interface, providing quantitative support for the dynamic bidirectional diffusion process in the RPA (glycerol)-CRISPR (sucrose) reaction system.

These findings prompted further investigation into how glycerol and sucrose enhance one-tube multi-enzyme reactions. As a disaccharide and a triol, respectively, sucrose and glycerol possess multiple hydroxyl groups capable of forming hydrogen bonds with water, leading to a three-dimensional HBN mediated by water bridging. We confirmed HBN formation in the dynamic bidirectional diffusion system via molecular dynamics (MD) simulations and temperature-dependent Raman spectroscopy. MD simulations showed that in 10% sucrose solution, increasing the temperature from 25 °C to 42 °C reduced hydrogen bond occupancy from 372.22% to 342.60%, with donor and acceptor atom counts decreasing by 16.3% and 13.18%, respectively (Fig. 3d). Raman spectra of 10% sucrose and glycerol solutions exhibited sub-peaks (S1 and S2) of hydrogen bonds stretching higher frequencies (cm^{-1}) at 42 °C (Fig. 3e-f). The energy of hydrogen bonds is correlated with their stretching frequencies, with higher frequencies corresponding to lower hydrogen-bond energies. Notably, the addition of Norovirus plasmids at different copy numbers produced consistent hydrogen-bonding trends, indicating that temperature-induced disruption of the hydrogen-bond network (HBN) occurred consistently across different target nucleic acid concentrations (Supplementary Fig. S9). To further investigate the influence of temperature on the sucrose HBN, additional control experiments were performed using pre-amplified RPA products. The completed RPA products were introduced into CRISPR/Cas12a systems containing either 10% sucrose or no sucrose, and fluorescence signals were monitored at both 25 °C and 42 °C (Supplementary Fig. S10). Because identical RPA products were used in all groups, variations arising from differences in RPA amplification efficiency were excluded. At 25 °C, the fluorescence intensity of the sucrose-free CRISPR system was approximately 2.76-fold higher than that of the sucrose-containing system, suggesting that the intact sucrose HBN restricted molecular diffusion within the reaction system. In contrast, at 42 °C, no significant difference in fluorescence intensity was observed between the two groups ($P > 0.05$), indicating that elevated temperature relaxed the sucrose HBN and alleviated diffusion constraints. These observations are consistent with temperature-induced relaxation of the sucrose HBN facilitating molecular transport. In addition, Cas12a retained detectable enzymatic activity throughout the 20–45 °C temperature range (RSD = 9.82%) in Supplementary Fig. S11, indicating that the observed fluorescence differences were primarily associated with changes in the sucrose matrix rather than loss of enzyme activity. Collectively, these results suggest that the RPA (glycerol)-CRISPR (sucrose) system operates through a synergistic diffusion-regulated mechanism involving temperature-responsive modulation of the sucrose hydrogen-bond network, rather than relying solely on concentration gradients or simple thermal diffusion. During the initial stage, glycerol increases local viscosity to effectively confine early amplification products. As the reaction proceeds at 42 °C, the elevated temperature provides optimal kinetic conditions for RPA and Cas12a activities while simultaneously inducing the partial relaxation of the sucrose hydrogen-bond network. Ultimately, the synergy between viscosity-driven phase maintenance and temperature-induced network relaxation facilitates the controlled bidirectional diffusion of nucleic acids and proteins, significantly enhancing overall signal amplification efficiency.

3.2. Ultra-sensitive detection of single copy level target by dynamic bidirectional diffusion-controlled multi-enzyme system

In this experiment, the conserved gene sequences of the Norovirus ORF1 and ORF2 junction regions were selected as detection targets to investigate the sensitivity and specificity (Fig. 4), and the intensity of fluorescence were used to evaluate the detection performance. For the dynamic bidirectional diffusion-controlled RPA/CRISPR-Cas12a assay, the fluorescence intensity performed a linear increase (Y = (7.2E + 0.6)x-(4.2E + 0.6), R^2 = 0.94) with the rise of the concentrations of GII Norovirus plasmid (Fig. 4a), and the LOD was 3.07 copies/μL, three replicates (100%) of each group tested positive [30]. For the conventional one-pot RPA/CRISPR-cas12a assay, the LOD was 3.07×10^2 copies/μL (Fig. 4b). Compared with the conventional one-pot method, our assay achieved a 100-fold improvement in detection sensitivity, reaching the single-copy level. Moreover, it exhibited a lower detection limit than the one-pot systems relying solely on sucrose or glycerol (10–100 copies) [28,32].Norovirus has an estimated infectious dose (ID_{50}) ranging from 18 to 1,320 copies [41,42]. Given that our assay achieves a LOD as low as 3.07 copies, we sought to further investigate whether the detection time could be shortened while still maintaining sufficient sensitivity to identify clinically relevant, infectious viral loads. Using the developed assay, Fig. 4c showed that the ID_{50} virus can be detected at 10–20 min, indicating our method can meet the practical application requirements and is suitable for on-site detection environments in a shorter detection time. In addition, our detection method has excellent specificity (Fig. 4d), only norovirus positive samples produce fluorescence signals, while Enterovirus EV71, Rotavirus and Adenovirus not produced significant fluorescence signals compared to blank samples. Subsequently, reproducibility and stability were assessed in 12 days (Supplementary Fig. S12), and ΔFL between positive samples and negative controls were calculated for coefficient of variation, and the CV = 4.62%, indicating good repeatability.

In recent years, nucleic acid detection technologies have developed rapidly, leading to the establishment of various detection platforms applied in clinical diagnostics, food safety, and other fields. The performance of nucleic acid detection assays reported in previous studies is summarized in Supplementary Table S2. In addition to relying on nucleic acid amplification strategies, some existing hydrogel-based sensing assays integrate smartphone-based image analysis and introduce artificial intelligence into the field of food detection. Although most of these methods have high sensitivity, there are some shortcomings. For example, LAMP and RPA methods often suffer from poor sensitivity, and dPCR requires large instruments and professional personnel. NASBA and CRISPR reaction temperatures and reagents are incompatible and require a two-step reaction. Compared with these assays, our work demonstrates high sensitivity, significantly reduces detection time, suitable for testing various types of food and clinical samples, does not rely on large instruments or equipment, and is therefore well suited for on-site detection applications.

3.3. Detection of clinical samples and food samples using all-in-one device

While the dynamic bidirectional diffusion-controlled system successfully enables one-pot amplification and detection, the POCT applications remain frequently bottlenecked by complex and time-consuming sample preparation steps. Hence, we integrated a custom 3D-printed nucleic acid extraction device with our multiphase system to establish an all-in-one device. This integration serves as a critical step to translate the one-pot assay, enabling the rapid processing of complex matrices (feces and food) without relying

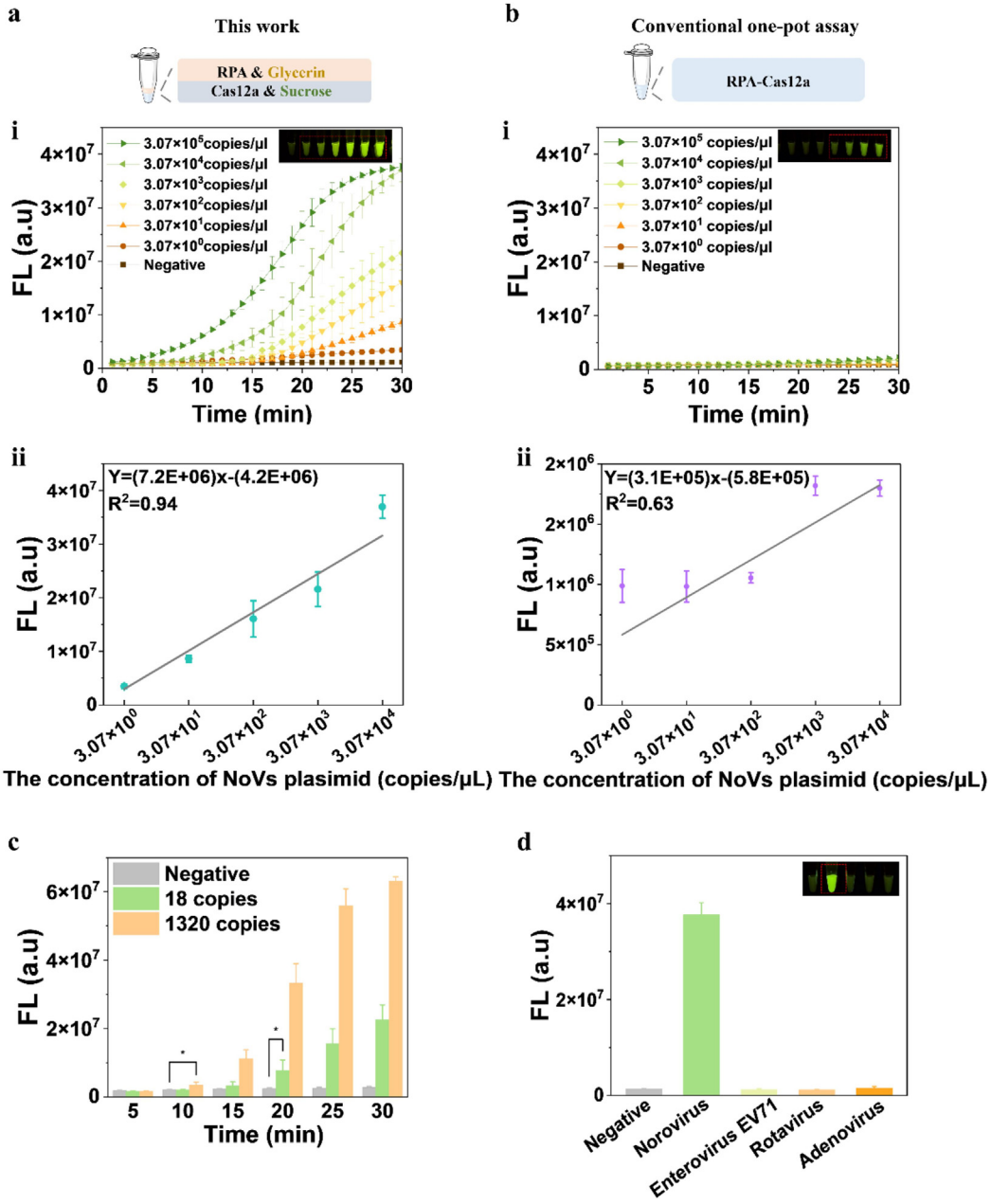


Fig. 4. Dynamic bidirectional diffusion-controlled multi-enzyme system detects Norovirus to single copy level. a) The sensitivity of dynamic bidirectional diffusion-controlled multi-enzyme system with different concentrations of GII Norovirus plasmid. NoVs: Norovirus. i: Fluorescence intensity. ii: Standard curve. b) The sensitivity of conventional one-pot RPA/CRISPR-cas12a assay with different concentrations of GII Norovirus plasmid. i: Fluorescence intensity. ii: Standard curve. c) The detection of ID_{50} Norovirus by dynamic bidirectional diffusion-controlled multi-enzyme system. d) The specificity of dynamic bidirectional diffusion-controlled multi-enzyme system.

on centralized laboratory equipment. A schematic diagram and dimensions of the all-in-one device were illustrated in Fig. 5a-b. The overall size of the device for manual sample preparation and nucleic acid extraction was 8.5 cm in length, and 2.4 cm in width. This device consisted of a cover plunger, span column, supporting pillar, and collection tube by using 3D printing for nucleic acid extraction and purification. As shown in Fig. 5a and Supplementary Fig. S13, the cover plunger pushed the liquid inside the span column into the collection tube. The span column with high-quality silica membrane as the adsorption membrane adsorbed nucleic acids through hydrogen bonding and electrostatic adsorption under high concentration ionization agents, while proteins substrates were not adsorbed and removed. The silica membrane, adsorbed nucleic acid, was washed to remove proteins and salts. Finally, the nucleic acid adsorbed on the silica membrane can be removed using RNase/DNase-free water, resulting in high purity of the nucleic acid. The supporting pillar supported the span column, and connected the collection tube. After washing and purity the nucleic acid, span column was transferred onto the detection tube and the nucleic acid was injected into dynamic bidirectional diffusion-controlled RPA/CRISPR-Cas12a system. Then, the all-in-one was device was used to detect the Norovirus in clinical fecal samples collected from Changchun Children's Hospital. After adding the sample to the reaction tube and incubating at 42 °C for 30 min, the target concentration can be directly inferred from the fluorescence output, enabling a simple and rapid detection process (Fig. 5b). As shown in Fig. 5c, our device successfully identified 19 out of 28 clinical samples as Norovirus-positive, exhibiting dis-

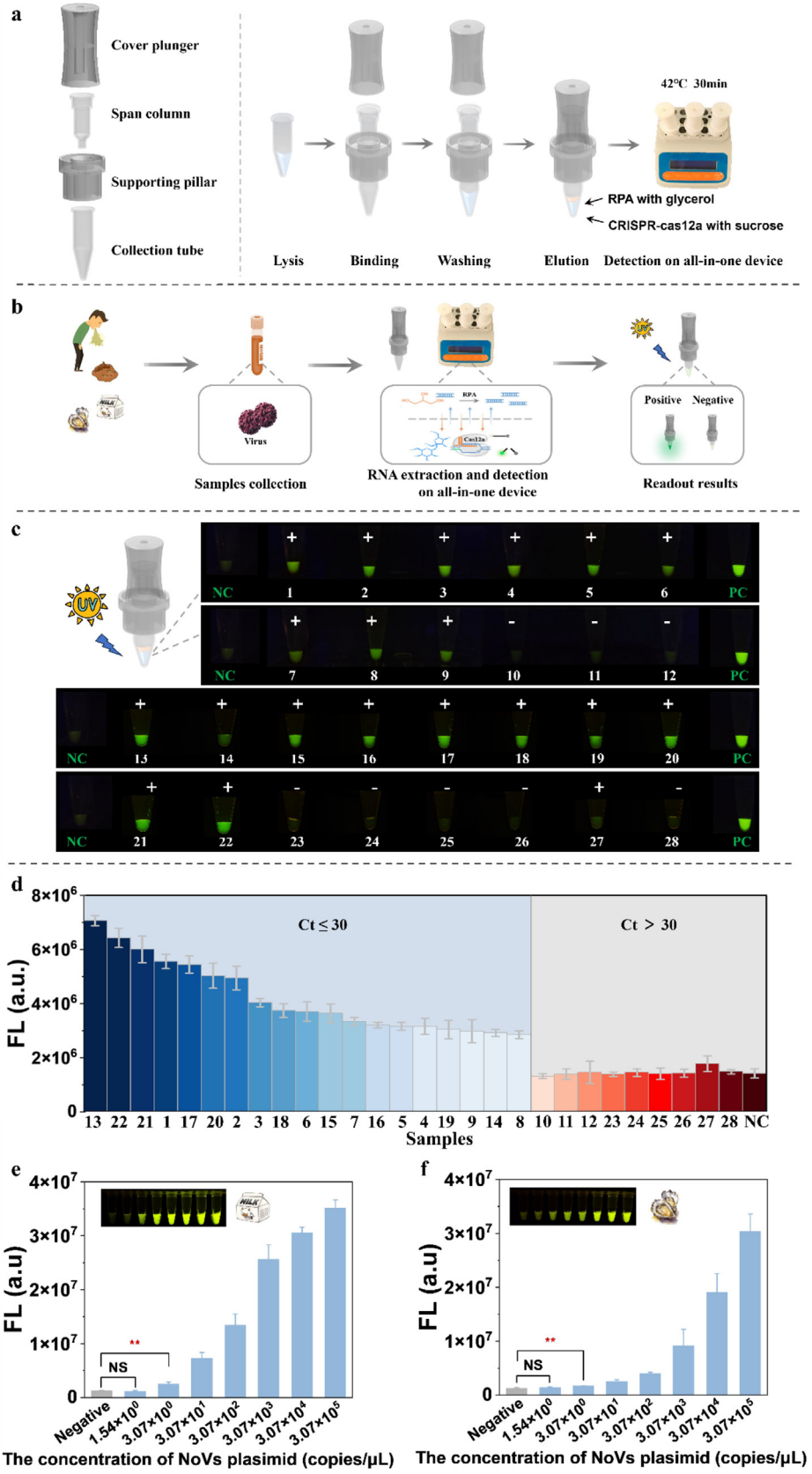


Fig. 5. Expanding dynamic bidirectional diffusion-controlled (RT)-RPA/CRISPR-Cas12a assay detection of target virus in complex samples. a) The exploded diagram and flow diagram of all-in-one device. b) Schematic of using all-in-one device for the detection of Norovirus. c) End point (30 min) signal images captured by smartphone for Norovirus detection in 28 clinical samples. -: Negative. + : Positive. NC: negative control. PC: positive control. d) The comparison of RT-qPCR and dynamic bidirectional diffusion-controlled RT-RPA/CRISPR-Cas12a assay. Negative samples are highlighted in gray, and positive samples are highlighted in blue. Relative fluorescence intensity obtained from the all-in-one device for detecting 28 clinical fecal samples. No.1-9, 13-22 were from patients with Norovirus. Adenovirus patients (No.10, 23-24), rotavirus patients (No.11, 25-26), and enterovirus patients (No.12, 27-28). e) The detection of Norovirus in milk samples. f) The detection of Norovirus in oyster samples.

tinct green fluorescence. The remaining nine samples showed fluorescence signals comparable to the blank control, indicating negative results. The detection results were consistent with those obtained by RT-qPCR method (Fig. 5d and Supplementary Fig. S14-15), which is the gold-standard for Norovirus nucleic acid detection. Although the preliminary clinical validation demonstrated an exceptional practicability (AUC = 1.00, Supplementary Fig. S15), it should be noted that this result is based on a relatively small sample size (n = 28). Future large-scale, multi-center trials are warranted to fully validate the robust clinical diagnostic performance of this platform. In addition to clinical samples, we evaluated the performance of the detection method in two food matrices: milk and oysters (Fig. 5e-f). Norovirus was not detected in either sample prior to spiking. Plasmid standards at varying concentrations (1.54, 3.07, 3.07 × 10^1, 3.07 × 10^2, 3.07 × 10^3, 3.07 × 10^4, and 3.07 × 10^5 copies/μL) were subsequently added, and detection was performed using the developed device with the dynamic bidirectional diffusion control multi-enzyme system. The LOD for both spiked milk and oyster samples reached 3.07 copies/μL, and the fluorescence intensity exhibited a linear increase (R^2 = 0.98–0.99) with the rise of the concentrations of GII Norovirus plasmids (Supplementary Fig. S16). These results demonstrating the method's strong applicability in complex food matrices. Furthermore, to evaluate the broad applicability of the proposed platform, we successfully applied this detection system to another common foodborne pathogen, *Salmonella* Typhimurium. The platform exhibited excellent detection performance consistent with traditional qPCR methods (Supplementary Fig. S17), demonstrating its robust versatility for diverse pathogenic targets.

4. Conclusion

In this study, we proposed a dynamic bidirectional diffusion-controlled RPA/CRISPR multi-enzyme system for one-pot detection. This system employs glycerol to accelerate the RPA reaction and sucrose to create a density gradient, effectively minimizing interference between enzymes the single-tube RPA/CRISP-Cas12a assay. This strategy addresses the compatibility issues of traditional one-pot assay and enable highly sensitive detection with a limit of 3.07 copies/μL. By integrating a 3D-printed nucleic acid extraction device, we established a sensitive and specific on-site detection method for Norovirus in both clinical and food samples. The results showed strong concordance with the gold-standard RT-qPCR method, while significantly reducing the detection time to 30 min, making it more suitable for POCT. The robust, modular, and portable hydrogen-bond based confined sensing platform further demonstrates strong potential for on-site food safety monitoring and scalable point-of-care testing of diverse pathogens. In future work, we aim to further integrate this system with smart phones, machine learning, and other digital technologies to develop a fully automated and intelligent virus detection platform.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2026.07.019.

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