



Improved Strategies for CRISPR-Cas12-based Nucleic Acids Detection

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Abstract

The COVID-19 pandemic has brought great challenges to traditional nucleic acid detection technology. Thus, it is urgent to develop a more simple and efficient nucleic acid detection technology. CRISPR-Cas12 has signal amplification ability, high sensitivity and high nucleic acid recognition specificity, so it is considered as a nucleic acid detection tool with broad development prospects and high application value. This review paper discusses recent advances in CRISPR-Cas12-based nucleic acid detection, with an emphasis on the new research methods and means to improve the nucleic acid detection capability of CRISPR-Cas12. Strategies for improving sensitivity, optimization of integrated detection, development of simplified detection mode and improvement of quantitative detection capabilities are included. Finally, the future development of CRISPR-Cas12-based nucleic acids detection is prospected.

Keywords CRISPR-Cas12 · Nucleic acid detection · Sensitivity · Integrated detection · Simplified detection mode · Quantitative detection

1 Introduction

The outbreak of COVID-19 poses a huge challenge to global public health security. Accurate and rapid screening of patients can effectively prevent the spread of such infectious diseases [1, 2]. Quantitative PCR (qPCR), as the gold standard of nucleic acids detection, has the characteristics of excellent accuracy, high sensitivity and high specificity. However, qPCR still requires a long turnaround time, expensive equipment and professional operators, which greatly limits its application in remote and resource-poor areas [3].

CRISPR is an adaptive immune mechanism widely existed in bacteria and archaea to defend against viruses and foreign nucleic acids [4]. CRISPR systems can be divided into two categories: class1 based on multiple protein effector complexes and class2 based on a single protein effector [5]. Among them, CRISPR-Cas12 is a class2 RNA-guided

CRISPR effector. Cas12 can be guided by its crRNA to recognize complementary single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) with protospacer-adjacent motif (PAM) sequences. Cas12 cleaves target DNA through a single RuvC domain [6]. In addition, the target DNA also can lead to the activation of the unique trans-cleavage activity of Cas12, which degrades ssDNA nonspecifically. When bound to an ssDNA target, LbCas12a-crRNA (Cas12a is a well-known subtype of Cas12) catalyzed trans-cleavage at a rate of 3 turnovers per second and a catalytic efficiency (k_{cat}/K_m) of $5.0 \times 10^6 \text{ s}^{-1} \cdot \text{mol}^{-1}$. When a bond to a dsDNA target, LbCas12a-crRNA catalyzed trans-cleavage at a rate of 17 turnovers per second and a catalytic efficiency of $1.7 \times 10^7 \text{ s}^{-1} \cdot \text{mol}^{-1}$ [7]. Therefore, efficient trans-cleavage can be used as an excellent signal amplification way for detection, and LbCas12a is able to detect DNA at pmol/L level using fluorophore/quencher-label ssDNA as trans-cleavage substrate [7, 8]. Besides, Cas12 is highly specific, requiring a 17 bp sequence match for stable binding and cleavage [9]. Overall, CRISPR-Cas12 has the advantages of signal amplification, high sensitivity and high specificity in nucleic acids detection. Although Cas12 only recognizes DNA, it also has the ability to detect RNA by incorporating reverse transcription. Since trans-cleavage activity was discovered in 2018, Cas12 has been used to develop a variety of nucleic acids detection platforms. Although vast quantities

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of CRISPR-Cas12-based nucleic acids technologies have been developed, their commercial application is still limited. Recently, research on CRISPR-Cas12-based nucleic acids detection system has improved dramatically. This paper reviews the progress in strategies for improving sensitivity, optimization of integrated detection, development of simplified detection mode and improvement of quantitative detection capacities. Finally, the future development of CRISPR-Cas12-based nucleic acids detection is prospected.

2 Strategies for Improving Sensitivity

Further improving the sensitivity of the Cas12 system is of great significance for nucleic acids detection. Nucleic acid amplification is an important way to improve the sensitivity, including PCR, recombinant polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP) and so on. These amplification technologies are highly sensitive, and the synergetic amplification of signal outputs can be achieved by Cas12 detection [10, 11]. Although amplification is widely used to enhance sensitivity, we believe that optimization of integrated detection is the key, which will be discussed in a later section. Therefore, this section focuses on two other ways of enhancing sensitivity, namely optimization of reaction buffer and crRNA engineering.

2.1 Optimization of Reaction Buffer

Appropriate reaction buffer can improve the sensitivity of Cas12 system by affecting the trans-cleavage activity. Cas12 utilizes the Mg^{2+} -dependent RuvC domain for cis- and trans-cleavage, so Mg^{2+} is almost a necessary component of Cas12 reaction buffers [7, 12]. However, Ma et al. [13] found that the reaction buffer containing Mn^{2+} was more suitable for LbCas12a system, and it produced the fluorescence signals 3.4–13.6-fold higher than the reaction buffer containing Mg^{2+} . On the contrary, some studies showed that the reaction buffer containing Mn^{2+} did not improve or even significantly reduce the sensitivity of Cas12a system

[14, 15]. In addition to changing the divalent cations in the reaction buffer, the addition of additives can also improve the sensitivity of Cas12 system. Polyethylene glycol (PEG), glycerin and Triton X-100 can improve the sensitivity of Cas12a system, but their mechanism of promoting trans-cleavage activity is unknown [16]. Li et al. [17] found that bovine serum albumin (BSA) could improve sensitivity of Cas12a system, while L-proline could protect Cas12a from an adverse environment. When BSA was added together with L-proline, L-proline improved sensitivity in the way of protecting BSA and Cas12a. In addition, appropriate pH enables sensitive Cas12 system. Yue et al. [15] found that LbCas12a had the highest trans-cleavage activity under weakly alkaline conditions (pH 9). Lv et al. [16] analyzed the trans-cleavage activity of LbCas12a in reaction buffers with pH ranging from 3.5 to 9.6 in increments of 0.1. Among the tested buffers, LbCas12a preferred buffers with pH 8.5 and 8.6. Currently, commercial NEBuffer 2.1 and NEBuffer 3.1 are often selected as reaction buffers for Cas12 in many studies, but optimized buffers in some studies can achieve a more sensitive Cas12 system than these two commercial buffers [14–16, 18, 19]. In general, the divalent cation, additive and pH are three important conditions that affect the sensitivity of the Cas12 system (Table 1 summarizes the components of the reaction buffers mentioned in this paragraph).

2.2 Optimization of crRNA Engineering

crRNA engineering was first applied to improve the efficiency and specificity of gene editing [20–24]. Recently, some studies have found that crRNA engineering can also improve the sensitivity of Cas12 system.

crRNA engineering can be achieved directly by extending the 3' or 5' ends of crRNA. Ooi et al. [25] found that crRNA with UA-rich 3' end extension could not improve the sensitivity of Cas12 system, while crRNA with a 9-nt 5' end extension might enhance sensitivity. However, such sensitivity improvement from 5' end extension may be accompanied by a higher background signal. In contrast to Ooi et al. [25],

Table 1 Component of Cas12 reaction buffers in different studies

Buffer No.	Component
Buffer 1	50 mmol/L Tris-HCl, 100 mmol/L NaCl, 10 mmol/L $MnSO_4$, 100 μ g/mL BSA, pH 7.9
Buffer 2	10 mmol/L Tris-HCl, 10 mmol/L NaCl, 15 mmol/L $MgCl_2$, 1 mmol/L DTT, 5% PEG-200, pH 9.0
Buffer 3	40 mmol/L Tris-HCl, 6 mmol/L $MgCl_2$, 1 mmol/L DTT, 2 mmol/L Spermidine, 40 mmol/L Glycine, 4% PEG-20000, 0.001% Triton® X-100, pH 8.5
Buffer 4	10 mmol/L Tris-HCl, 50 mmol/L NaCl, 10 mmol/L $MgCl_2$, 100 μ g/mL BSA, pH 7.9
Buffer 5	50 mmol/L Tris-HCl, 100 mmol/L NaCl, 10 mmol/L $MgCl_2$, 100 μ g/mL BSA, pH 7.9

Buffer 1, used in Ref. [13]. Buffer 2, used in Ref. [15]. Buffer 3, used in Ref. [16]. Buffer 4, known as NEBuffer 2.1. Buffer 5, known as NEBuffer 3.1

Nguyen et al. [14] concluded that crRNA with UA-rich 7-nt or 13-nt 3' end extension could significantly improve sensitivity, while crRNA with UA-rich 5' extension had little effect on sensitivity.

crRNA engineering can also be achieved by introducing DNA. crRNA containing two or four DNA base substitutions could improve sensitivity significantly [25]. In addition, crRNA with TA-rich 7-nt DNA 3' end extension also significantly improved sensitivity, and its fluorescence signal was 3.5-fold higher than the wild-type crRNA. However, such crRNA could improve the sensitivity of LbCas12a and FnCas12a, but reduce the sensitivity of AsCas12a [14]. Shi et al. [26] designed switchable-caged guide RNA (scgRNA) with self-reporting capability and developed CRISPR-Cas-only amplification network (CONAN). scgRNA consisted of two DNA fluorescent probes and one crRNA. In CONAN reaction, ssDNA bulges in scgRNA were degraded as trans-cleavage substrate, which resulted in the production of fluorescent signal and the release of blocked crRNA. The released crRNA guided Cas12a to recognize the target DNA and also cleaved ssDNA bulges in scgRNA via trans-cleavage activity. Through this positive feedback circuit, CONAN achieved a sensitivity of amol/L level.

Compared with natural crRNA, chemically modified crRNA is more stable and, therefore, has equal or stronger editing efficiency [21, 23, 24]. Surprisingly, crRNA modified with 2'-O-methyl RNA bases, 2'-fluoro bases and phosphorothioate linkages could significantly improve the sensitivity of Cas12 system [25]. However, when the 3' and 5' end of crRNA were extended with phosphorothioate DNA, respectively, the sensitivity of Cas12 system decreased significantly. Although crRNA with TA-rich 7-nt DNA 3' end extension had higher sensitivity, its sensitivity would decrease with the addition of more phosphorothioate modification to the extension [14].

The above three crRNA engineering methods can improve the sensitivity of Cas12 system. However, there are conflicting conclusions of crRNA engineering from different studies. This may mean that this crRNA engineering method is not universal and may be limited by Cas12 type, reaction conditions and crRNA sequence.

3 Optimization of Integrated Detection

The combination of amplification reaction and Cas12 detection reaction can achieve sensitive detection. However, the extra cap-opening process between amplification and detection inevitably increases the risk of carryover contamination [10]. Integrated detection, which integrates amplification reaction and detection reaction in a closed environment, avoids contamination and simplifies operation procedures. However, amplification reaction and detection reaction

interfere with each other. The target DNA may be degraded by Cas12 at the initial stage of the reaction, resulting in inefficient detection. In addition, different reaction temperatures limit the integration of amplification reaction and detection reaction. Therefore, many studies are focusing on optimizing integrated detection.

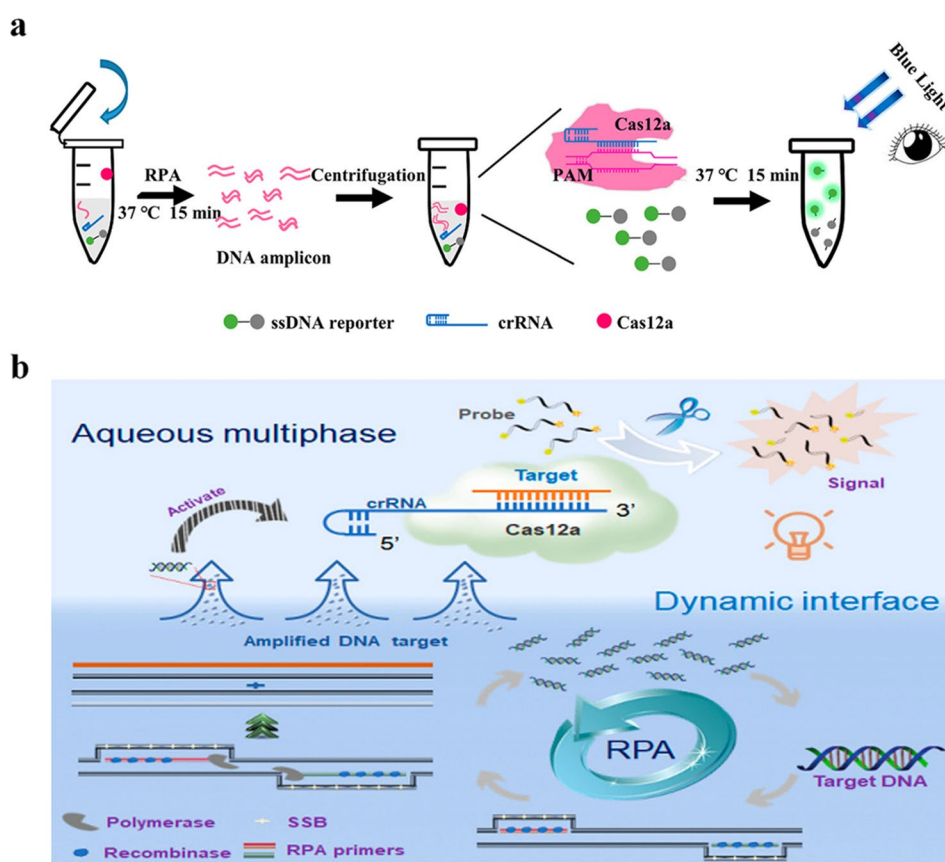
3.1 Integrated Detection Based on Spatial-temporal Separation

Integrated detection based on spatial-temporal separation separates amplification reaction and detection reaction in a closed system, thus effectively avoiding interference between the two reactions. Wang et al. [27] added Cas12a detection reagent to the tube wall and RPA reagent to the tube bottom. After the amplification reaction, the detection reagent was mixed with the amplification products by centrifugation (Fig. 1a). In addition, Cas12a reagent could be added to the tube cap. When LAMP at high temperature was finished at the bottom of the tube, the detection reagent was mixed with the amplification products [28, 29]. Simple devices could also be used for integrated detection, which preformed amplification reaction and detection reaction in different chambers. After amplification reaction, the amplification products were transferred to the detection chamber by simple operation [30–32]. However, the spatial-temporal separation between amplification reaction and detection reaction can be less rigid. Yin et al. [33] constructed a dynamic aqueous multiphase reaction (DAMR) system by using various concentrations of sucrose solutions. In DAMR system, RPA reaction was located at the bottom phase with high density, while Cas12a detection reaction was located at the top phase with low density. This spatially separated but connected aqueous phases allow RPA amplification products to gradually diffuse with the top phase and activate the detection reaction (Fig. 1b). In general, integrated detection based on spatial-temporal separation ensures that amplification reaction is not disturbed, but an additional operation step is required to begin Cas12 detection.

3.2 Integrated Detection Based on Spatial-temporal Consistency

Integrated detection based on spatial-temporal consistency does not separate amplification reaction from detection reaction, so ingenious schemes or system optimization need to be introduced to avoid interference. The reaction temperature of RPA and recombinase-aided amplification (RAA) is similar to that of Cas12a detection, which is conducive to the realization of integration. Ding et al. [34] constructed an all-in-one dual CRISPR-Cas12a (AIOD-CRISPR) assay without PAM sequence limitation by integrating RPA reaction and Cas12a detection reaction. Dual Cas12a-crRNA complexes

Fig. 1 Integrated detection technology based on spatial–temporal separation. **a** Schematic of a CRISPR-Cas12-based nucleic acids detection technology integrating RPA with Cas12 detection in a single reaction system. Adapted from Ref. [27]. Copyright 2019 American Chemical Society. **b** Schematic of dynamic aqueous multiphase reaction (DAMR) system. Adapted from Ref. [33]. Copyright 2020 American Chemical Society



would recognize ssDNA and activate trans-cleavage activity when ssDNA was exposed by a strand displacement reaction. In this strategy, the selection of crRNAs without PAM sequence limitation was a key since crRNA with PAM sequence would result in the cleavage of templates, thus reducing the amplification efficiency of RPA [10]. To minimize the interference of Cas12 detection on amplification, Chen et al. [35] reduced the dosage of Cas12a. The detection limit was consistent with RAA when the concentration of Cas12a was 25 nmol/L. Unlike RPA and RAA, LAMP performs at a temperature of 60–65 °C, which is not compatible with Cas12 detection reaction. In order to solve this problem, integrated detection based on LAMP appropriately reduced reaction temperature and selected thermophilic Cas12b or modified enAsCas12a [19, 25, 36]. Dual-priming isothermal amplification (DAMP) was an isothermal amplification technology based on LAMP, which adopted a new primers design strategy and had the advantages of shorter amplification time, higher sensitivity and lower non-specific signal [37]. Ding et al. [38] constructed one-pot warm-start CRISPR (One-pot WS-CRISPR) assay by combining RT-DAMP. To solve the problem of reaction temperature incompatibility, one-pot WS-CRISPR lowered the reaction temperature to 52 °C by reducing the concentration of Mg^{2+} and using phosphorothioate inner primers. However,

both cis- and trans-cleavage are Mg^{2+} -dependent, and a low concentration of Mg^{2+} may lead to inefficient detection reaction [7, 12]. Therefore, one-pot WS-CRISPR selected Alt-R Cas12a *Ultra* and added pyrophosphatase. Alt-R Cas12a *Ultra* could work at low concentrations of Mg^{2+} , while pyrophosphatase could degrade the magnesium pyrophosphate precipitates generated during amplification and maintain a constant concentration of Mg^{2+} . In general, many studies have achieved integrated detection based on spatial–temporal consistency by optimizing sample treatment, adjusting component concentration, adopting more efficient primers and screening efficient fluorescent probes [34, 39–41]. Although integrated detection based on spatial–temporal consistency achieves sensitive detection, it cannot completely avoid interference between the reactions, which may cause the loss of detection sensitivity.

4 Development of Simplified Detection Mode

Traditional CRISPR-Cas12-based nucleic acids detection is mainly based on fluorescence methods, but the fluorescence methods require additional device assistance [7, 19, 36, 41–43]. In contrast, nanoparticle-assisted

CRISPR-Cas12-based nucleic acids detection does not require an additional device, providing a simplified detection mode.

4.1 Lateral Flow Assay

Lateral flow assay (LFA) is a rapid, cheap and simple paper-based technology that indicates detection results by capturing gold nanoparticles (AuNPs) on test line and control line [44]. Cas12 detection can be integrated with LFA as signal output strategy to achieve a simplified detection mode. Some LFAs display detection results in signal-on mode. The modified ssDNAs could capture all AuNPs on the control line when there were no targets. Once the Cas12 system was activated, cleavage of modified reporter probes would reduce accumulation at the control line and result in signal on test line [36, 40, 45, 46]. Some studies have constructed LFA working in signal-off mode. Biotinylated ssDNAs could hybridize with complementary capture probes immobilized on the test line. Activated Cas12 cleaved biotinylated ssDNA, resulting in AuNP-streptavidin conjugates not being captured at the test line. Therefore, positive result exhibited no test line while negative result exhibited a test line [47,

48] (Fig. 2a). Overall, LFA is a powerful diagnostic tool that provides intuitive diagnostic results.

4.2 Visual Colorimetric Assay and Microfluidic Chip Assisted Assay

Visual colorimetric assay utilizes the physical properties of nanoparticles to achieve a simplified detection mode. Some studies used ssDNA to control the aggregation and dispersion of AuNPs and indicated the detection results by visual color changes. One strategy used ssDNA to cross-link DNA-AuNP probe pairs. Degradation of ssDNAs due to trans-cleavage would result in de-cross-linking DNA-AuNP probe pairs and color changes. This method could improve the signal-to-noise ratio of the detection results by low-speed centrifugation [49, 50]. Jiang et al. [51] used a biotinylated ssDNA which could bind to streptavidin-coated magnetic beads and hybridize with a DNA-AuNP probe. When ssDNAs were degraded, DNA-AuNP probes could not be pulled down by streptavidin-coated magnetic beads. As a result, the supernatant solution showed the red color DNA-AuNP probes [52] (Fig. 2b). Zhang et al. [53] directly used DNA-AuNP probes as trans-cleavage substrates. Once

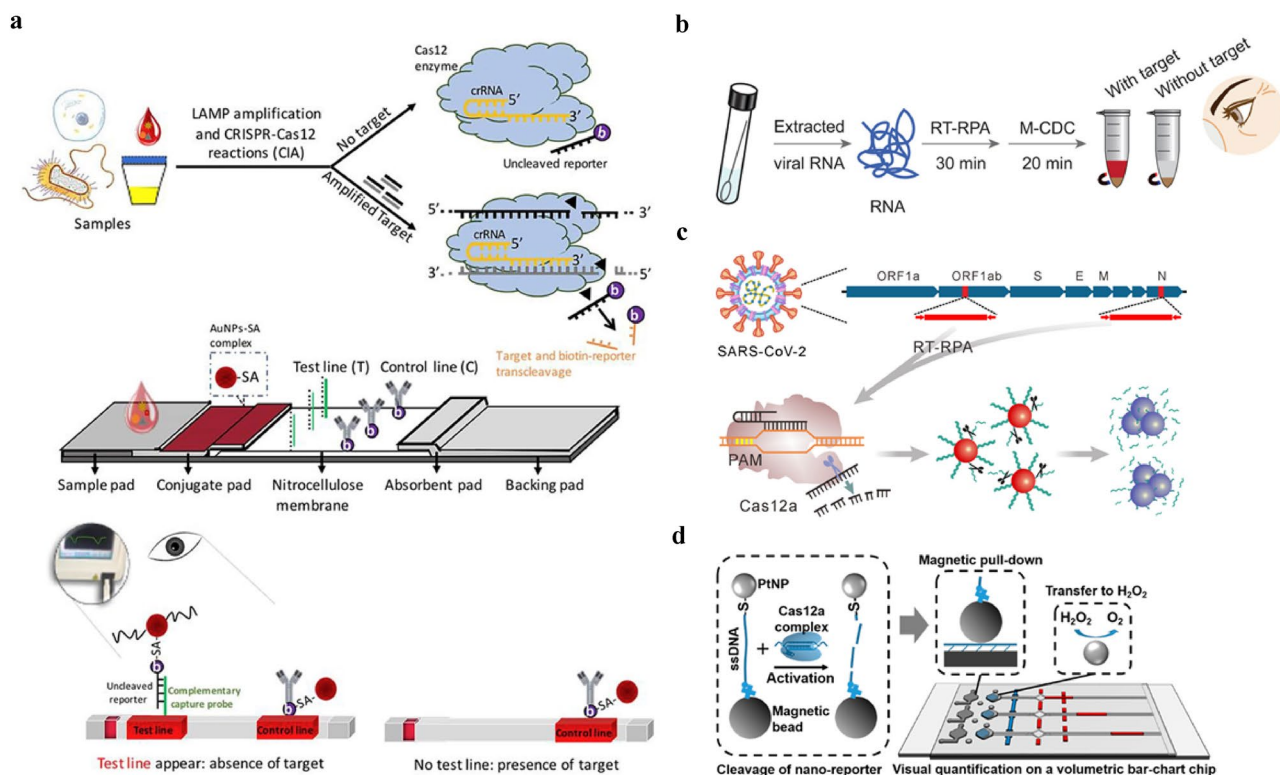


Fig. 2 Nanoparticle-assisted CRISPR-Cas12-based nucleic acids detection. **a** Schematic of LFA which works in signal-off mode. Adapted from Ref. [48]. Copyright 2020 Elsevier. **b** Schematic of a magnetic pull-down-assisted colorimetric method. Adapted from Ref. [51]. Copyright 2021 American Chemical Society. **c** Schematic

of colorimetric analysis using DNA-AuNP probes as trans-cleavage substrates. Adapted from ref. [53]. Copyright 2021 American Chemical Society. **d** Schematic of CRISPR-Cas12a coupled with PtNP for visual detection on a volumetric bar-chart chip. Adapted from Ref. [54]. Copyright 2019 American Chemical Society

trans-cleavage activity was activated, the capped DNA substrate would be degraded from AuNPs, leading to a decreased and red-shifted surface plasmon resonance peak along with AuNP aggregation (Fig. 2c).

The combination of microfluidic chip and nanoparticle provides a new strategy for simplified detection. Shao et al. [54] integrated platinum nanoparticle (PtNP) and volumetric bar-chart chip to realize the volumetric quantification of nucleic acids. In this technology, PtNP was part of magnetic bead-ssDNA-platinum nanoparticle (BDNP) reporter. Activated CRISPR-Cas12 cleaved the ssDNA of BDNP reporters, resulting in the release of PtNPs. The released PtNPs were no longer pulled down by the magnet and reacted with H_2O_2 to generate oxygen. The advancements of the red ink in the channels, propelled by oxygen, indicated the corresponding amount of target DNA (Fig. 2d).

In general, these technologies simplify detection mode and achieve visual detection without external devices. However, these technologies generally require complicated operation procedures, which is not conducive to the development of point-of-care testing (POCT) detection method.

5 Improvement of Quantitative Detection Capabilities

Digital polymerase chain reaction (dPCR) provides an absolute quantitative technology for nucleic acids detection, but its application is limited by long detection time and heavy

thermal cycling equipment. CRISPR-Cas12 detection provides a new way for quantitative detection. Some CRISPR-Cas12-based quantitative detection technologies require a miniature heater and can replace fluorescence microscopy with mobile phone-based fluorescence detection. Therefore, CRISPR-Cas12-based quantitative detection has a certain advantage.

5.1 Quantitative Detection Based on Isothermal Amplification

Isothermal amplification has the advantage of shorter reaction time and constant reaction temperature, but it is susceptible to false positives. The combination of isothermal amplification and CRISPR-Cas12-based nucleic acids detection achieves a second recognition step for amplification products, which has the potential to build a rapid, sensitive and accurate quantitative detection platform. Digitization-enhanced CRISPR/Cas-assisted one-pot virus detection (deCOViD) and rapid digital CRISPR approach (RADICA) were two quantitative detection technologies taking advantage of AIOD-CRISPR [55, 56] (Fig. 3a). However, in order to avoid undesired premature target amplification at room temperature, quantitative detection based on RPA needs to prepare all reaction mixture on ice and loads into the chip within 1 min after adding Mg^{2+} , which makes the detection procedure more complex [56]. Therefore, Ding et al. [38] solved this problem by increasing the initiation temperature of amplification, and constructed digital warm-start CRISPR

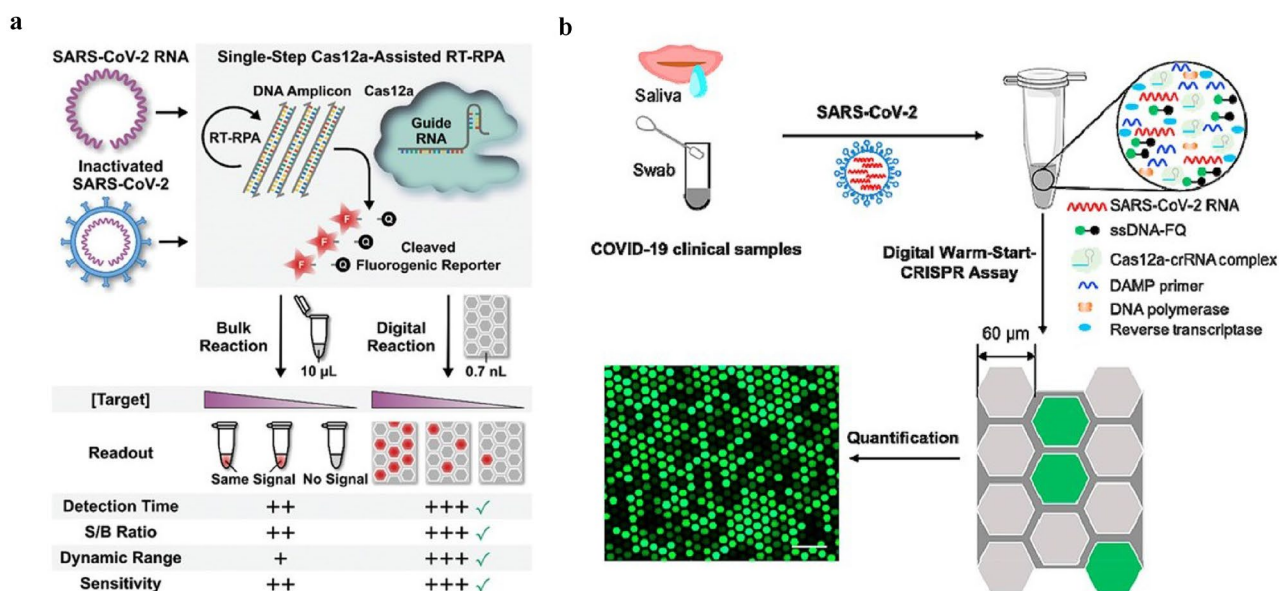


Fig. 3 Quantitative detection technologies based on isothermal amplification. **a** Schematic of digitization-enhanced CRISPR/Cas-assisted one-pot virus detection (deCOViD). Adapted from Ref. [55]. Copy-

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(dWS-CRISPR) based on one-pot WS-CRISPR (Fig. 3b). In general, the above three technologies have the advantages of high specificity, short detection time and isothermal reaction over dPCR.

5.2 Amplification-free Quantitative Detection

Quantitative detection based on isothermal amplification has the risk of contamination, so it is important to develop amplification-free quantitative detection. Although amplification-free quantitative detection has the potential to address the problem of false positive, it faces the problem of a low signal-to-noise ratio. There are two solutions: (1) improving the sensitivity by optimizing the Cas12 system and introducing multiple crRNAs, and (2) improving the target concentration by reducing the volume of droplet. Yu et al. [57] achieved quantitative detection by optimizing the ssDNA detection system and designing a microfluidic chip with 3 pL microwells. However, this technology is not suitable for most detection scenarios because it is limited to ssDNA detection. Our group had comprehensively optimized Cas12a system, which could directly detect DNA at 100 fmol/L level in bulk solution. In addition, we have designed a microfluidic chip capable of producing droplets with a diameter of 20 μm and adopted a dual-crRNA targeting strategy to improve detection efficiency. By combining all the optimization conditions, our team finally achieved efficient and sensitive quantitative detection [15] (Fig. 4). Although amplification-free quantitative detection has significant advantages, all reactions need to be prepared at low temperature, which is not conducive to their application.

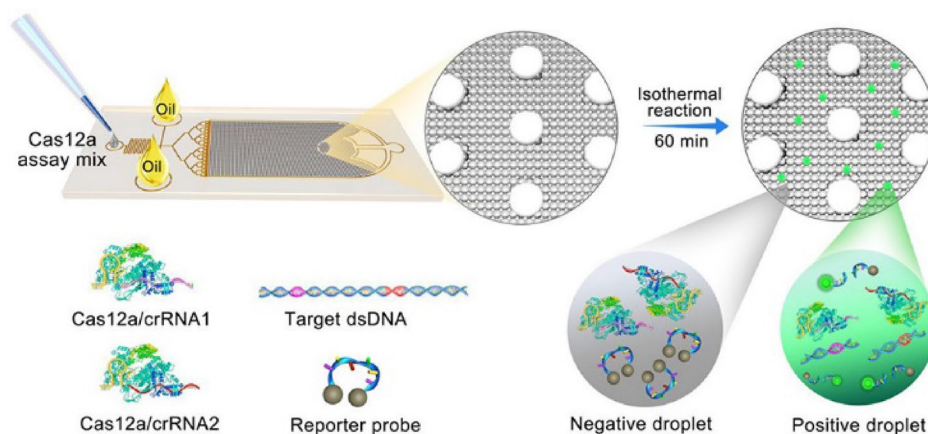
6 Conclusions and Outlook

In this review, we discussed recent advances in CRISPR-Cas12-based nucleic acids detection from four aspects. First of all, optimization of reaction buffer and crRNA

engineering can significantly improve the sensitivity of Cas12 system. The improvement of sensitivity is related to the mechanism of Cas12. A recent study reported a startling discovery that AsCas12a programmed with split crRNAs catalyzed cis-cleavage and trans-cleavage [58]. This mind-boggling finding suggests that many unknown properties of Cas12 remain to be studied. Therefore, exploring the properties of Cas12 may provide a new approach to improve the sensitivity of Cas12 system. In addition, the sensitivity of Cas12 system may be improved by Cas12 protein engineering and new fluorescent probes. Secondly, several strategies are applied to optimize integrated detection and two types of integrated detection are constructed. However, both types of integrated detection have some drawbacks, so more convenient and high-sensitivity integrated detection technologies need to be developed. In the future, integrated detection can also integrate simple sample processing methods to develop commercial integrated detection devices. Third, nanoparticle-assisted CRISPR-Cas12-based nucleic acids detection provides a simplified detection mode. These technologies possess the excellent qualitative diagnostic ability, but some of them still need to improve their quantitative diagnostic ability. Furthermore, these technologies need to simplify the operation process and improve the signal-to-noise ratio, which is conducive to POCT. Finally, CRISPR-Cas12-based quantitative detection technologies were developed, providing a powerful tool for clinically precise diagnosis. However, these technologies still have some defects and need further improvement. In the future, the development of CRISPR-Cas12-based quantitative detection technology for multiplexed DNA and single base mutation has broad development prospects.

CRISPR-Cas12-based nucleic acids detection has gradually developed towards commercialization. By combining engineering, materials and other disciplines, CRISPR-Cas12-based nucleic acids detection can be further developed. We believe that CRISPR-Cas12 based nucleic acids detection technology in the future can not only achieve

Fig. 4 Schematic of amplification-free quantitative detection. Adapted from Ref. [15]. Copyright 2021 American Chemical Society



amplification-free detection with high sensitivity and high specificity but also have the advantages of user-friendliness and low cost.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

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