Preamplification and Amplification-Free Detection of SARS-CoV-2 based on CRISPR/CAS SYSTEM

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ABSTRACT

Since the end of December 2019, the outbreak of COVID-19 caused by severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) infection has been occurring worldwide. SARS-CoV-2 is highly contagious. Rapid and accurate detection of SARS-CoV-2 is the key to interrupting the spread of the disease and preventing the epidemic. Recently, the novel molecular diagnostic tools based on CRISPR/Cas system have attracted more and more attention in the field of nucleic acid detection. In this paper, we summarized the relevant studies on the CRISPR/Cas system for rapid diagnosis of SARS-CoV-2, and compare pre-amplification assisted detection methods with amplification-free methods based on CRISPR/Cas system on the limit of detection and detection time, which is of great significance for further optimizing the CRISPR/Cas system-based SARS-CoV-2 detection technology.

Keywords: CRISPR/Cas; SARS-CoV-2; Preamplification; Amplification Free

Introduction

Severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) is a single-stranded positive strand RNA virus, belonging to Beta corona virus family [1,2]. Due to the strong infectivity and lethality of SARS-CoV-2, the detection of SARS-CoV-2 with rapid, accurate and convenient diagnostic methods is the key to find the source of infection, intercept the chain of disease transmission and then prevent and control the epidemic [3]. Commonly used pathogen detection methods include pathogen culture, antibody detection, nucleic acid detection, etc., among which virus culture detection takes several days and is too inefficient. Serum antibody tests are less specific [4]. So far, the most used detection method in clinical practice is the nucleic acid detection method with shorter time, stronger specificity and higher sensitivity. At present, there are several kinds of nucleic acid detection methods for SARS-CoV-2, such as polymerase chain reaction (PCR), isothermal amplification, etc. [4,5]. Clustered regularly interspaced short palindromic repeats (CRISPR) and its related proteins are an RNA-guided adaptive immune system in microorganisms. CRISPR is a unique family of forward repeats [6,7]. In recent years, CRISPR technology has developed rapidly, not only widely used in gene

editing, but also attracted more and more attention in the field of nucleic acid detection [8,9]. This review summarizes the current methods for detecting SARS-CoV-2 based on the CIRSPR/Cas system and compares pre-amplification-based detection methods with amplification-free detection methods on the limit of detection and detection time.

Brief Introduction of the Workflow in CRISPR/Cas based SARS-COV-2 Detection

Figure 1 presents the common workflow in CRISPR/Cas based SARS-COV-2 detection. First, nasal samples or saliva samples are collected from the patients [1]. Then, viral RNA is extracted from the sample. After the RNA extraction, reverse transcription reaction is carried out according to the Cas protein used. For Cas13 based system, reverse transcription is not required while for Cas12 based system, reverse transcription is required. Then, optional preamplification step is adopted before the CRISPR/Cas reaction to increase the concentration of the target. Recently, preamplification free methods have also been adopted in several studies, to simplify operation steps and shorten reaction time. Afterward, Cas protein and CRISPR/Cas RNA (crRNA) complex recognize specific regions of target molecules. The Cas protein is activated upon bounding and cleaves the reporter molecular, resulting in signal development which can be detected by the signal readout system [10].

Many Cas protein such as Cas9 [11], Cas12 [12], Cas13 [13] and Cas14 [14] have been utilized in nuclear acid detection. Among them, Cas12 and Cas13 are mostly commonly used [10,15]. Cas12 protein includes Cas12a and Cas12b, is guided by crRNA to bind to the complementary DNA strand with T-rich PAM sequence (5'-TTTV -3'). Cas12 can be activated without trans-activating crRNA. Besides, Cas12 could cleave the non-target DNA once activated by the target DNA sequence, which can be used for signal amplification in detection [16,17]. Cas13 protein is another kind of CRISPR protein which targets RNA. Its cleavage activity is activated by single strand RNA with complementation of its crRNA spacer sequence. After activated, Cas13 can cleave any adjacent single strand RNA. Based on this feature, another high -sensitive, high -specific molecular diagnosis method is established [12,18,19].

Preamplification Assisted Detection Methods based on CRISPR/Cas System

When the target concentration is lower than 10 nM, it is difficult to detect samples quickly and accurately using nucleic acid detection method based on CRISPR/cas system [15,20]. Therefore, different amplification methods, for example, PCR [21,22], LAMP [23], and RPA [5] have been utilized to enhance their sensitivity (Figure 2). For example, RPA was used to amplify the target concentration and increased sensitivity by 6 orders of magnitude in SHERLOCK system [5,24]. In another system based on Cas12a, the system sensitivity is improved by 7 orders of magnitude by using 45-minute RT-LAMP pre amplification [23]. Preamplification method such as PCR is more sensitive than RPA. However, PCR requires sophisticated thermal cycling, making the equipment more complex. Besides, the reaction temperature of PCR is not compatible with the CRISPR/Cas reaction. As a result, PCR based CRIPSR/Cas detection method is restricted in one pot reaction. Isothermal amplification methods such as RPA and LAMP avoid the thermal cycling procedures, and the reaction temperature is more compatible with CRISPR/Cas reaction. Therefore, preamplification and CRISPR reaction can be combined in one pot, making this method more suitable for rapid clinical detection. Compared with RPA, LAMP needs 4 to 6 primers for sequence amplification and the primer design was limited by the PAM and PFS regions in CRISPR reaction. Therefore, it is more difficult to design LAMP primers than RPA primers. Complex primer design may also result in false positive signal in LAMP reaction. Therefore, RPA is the most appropriate preamplification method combined with CRISPR analysis (Table 1).

Table 1: Different preamplification methods used in CRISPR/Cas system.

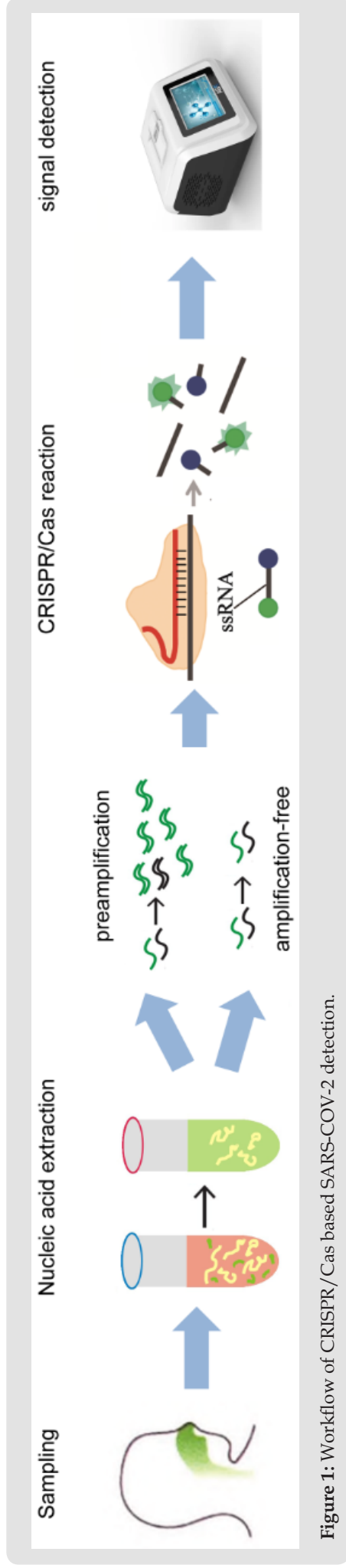|	Reaction temperature	One pot reaction	Primer design	Sensitivity	Specificity	Instrument complexity
RPA	37 °C	Yes	2 primers	+	++	Simple, cost-effective
LAMP	60 °C	Yes	4-6 primers	+	++	Simple, cost-effective
PCR	60-90 °C	No	2 primers	++	+	complex

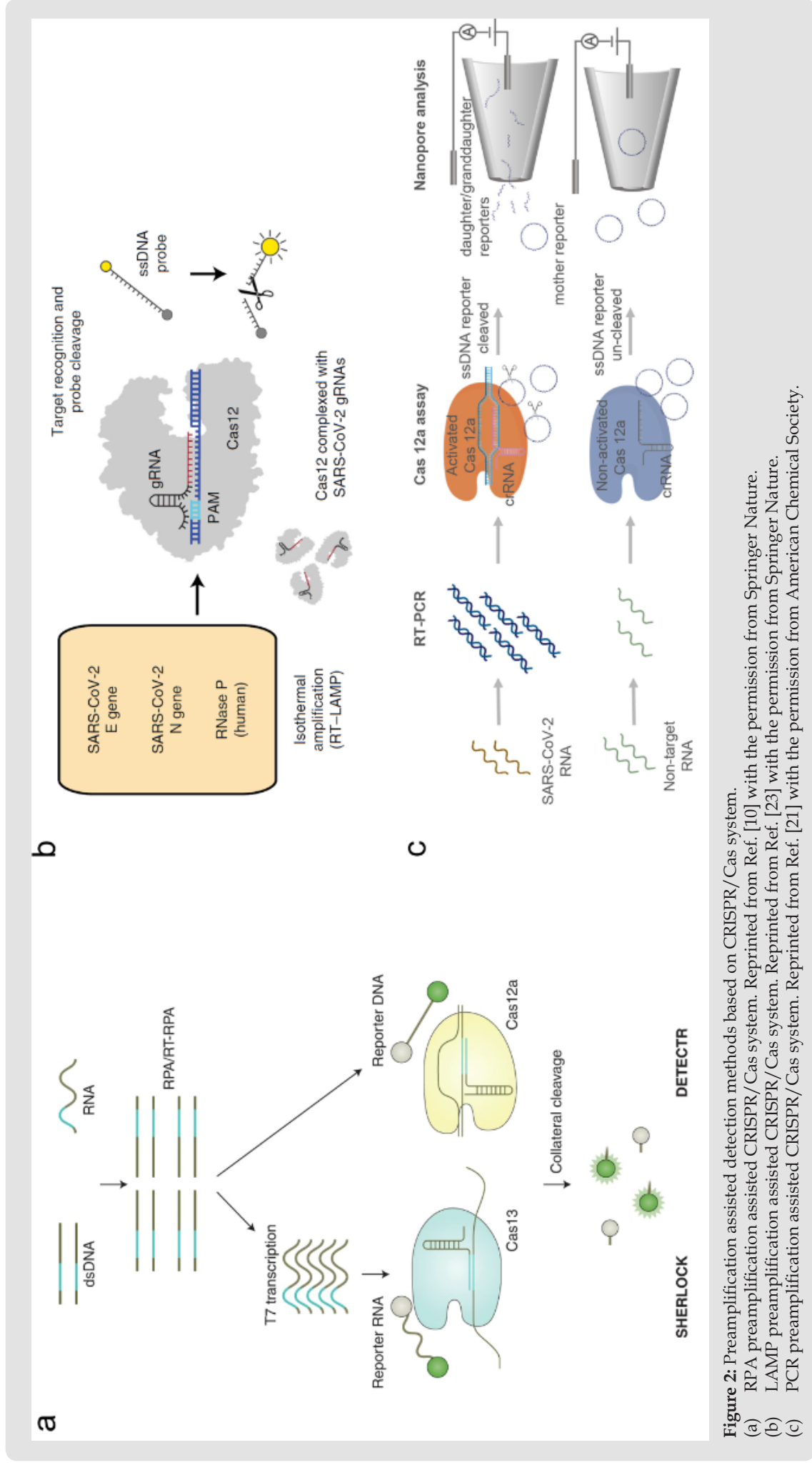**Figure 1:** Workflow of CRISPR/Cas based SARS-COV-2 detection.

Figure 2: Preamplification assisted detection methods based on CRISPR/Cas system.

(a) RPA preamplification assisted CRISPR/Cas system. Reprinted from Ref. [10] with the permission from Springer Nature.

(b) LAMP preamplification assisted CRISPR/Cas system. Reprinted from Ref. [23] with the permission from Springer Nature.

(c) PCR preamplification assisted CRISPR/Cas system. Reprinted from Ref. [21] with the permission from American Chemical Society.
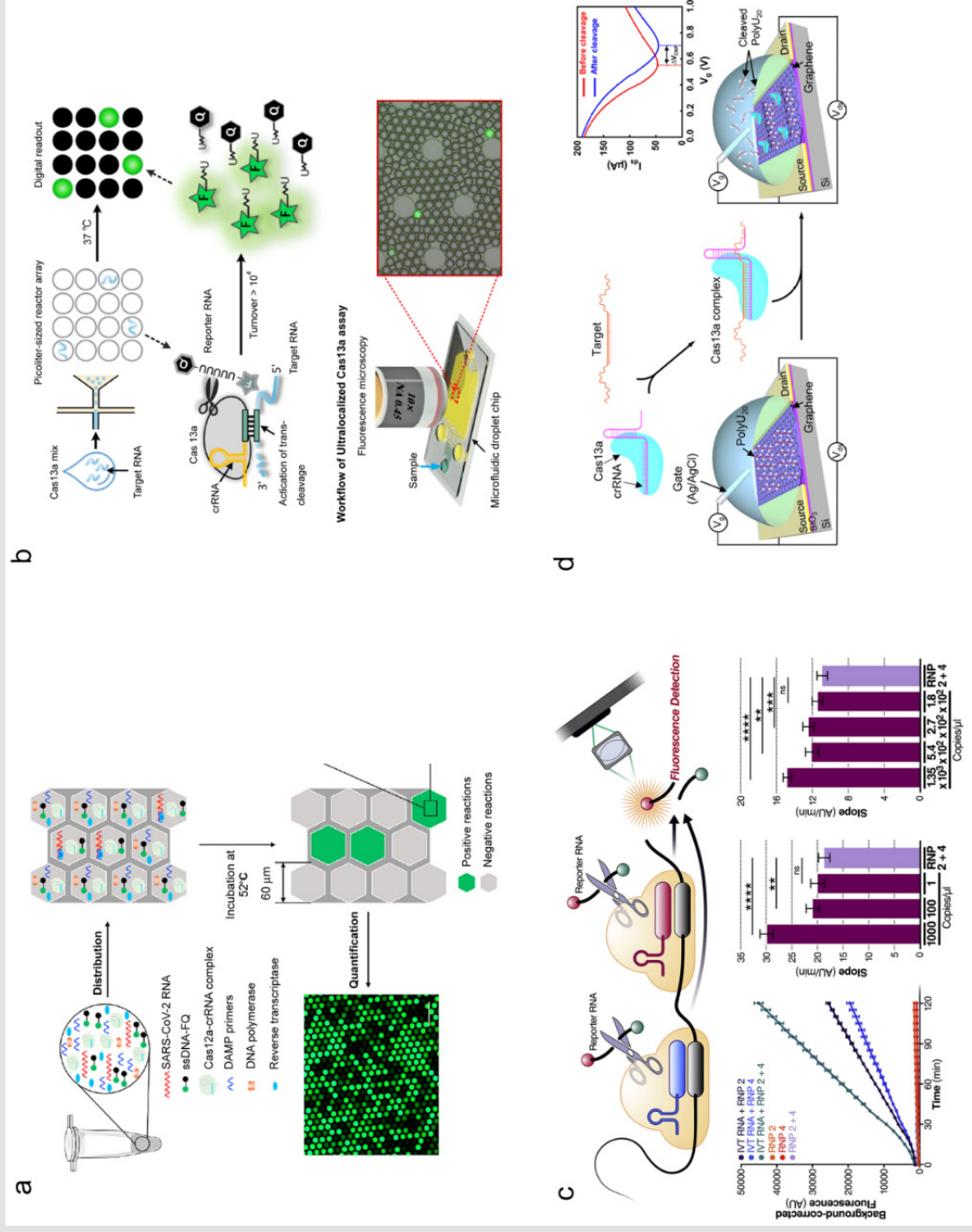
Figure 3: Amplification-free detection methods based on CRISPR/Cas system.

(a) Digital CRIPSR for amplification-free SARS-COV-2 detection. Reprint from Ref. [43] with the permission from Elsevier.

(b) Droplet digital CRIPSR for amplification-free SARS-COV-2 detection. Reprint from Ref. [27] with the permission from American Chemical Society.

(c) Amplification-free SARS-COV-2 detection using multiple crRNA. Reprint from Ref. [28] with the permission from Elsevier.

(d) Amplification-free SARS-COV-2 detection using sensitive readout system. Reprint from Ref. [30] with the permission from Wiley.

Amplification-Free Detection Methods based on CRISPR/Cas System

Although the sensitivity of the CRISPR/Cas system could be improved by preamplification, the preamplification steps increase the cost and complicated the system design. To resolve the problem, several amplification-free detection methods have been developed. The commonly used strategies are digital CRISPR, the use of multiple crRNA and sensitive readout system (Figure 3) [25,26]. In CRISPR reaction, the decrease of reaction volume will lead to the increase of the concentration of cracking reporter. Therefore, reducing the reaction volume will improve the sensitivity of the system. Some recent studies have improved the analytical performance by allocating the reaction system to many droplets which has been used in digital PCR. For example, Tian, et al. [27] have greatly improved the detection sensitivity of the CRISPR/Cas system by using droplets of 15 pL as reaction carriers [27]. Another advantage of digital analysis is that absolute target quantification can be carried out without standard curve.

Another amplification free method is to use multiple crRNA to improve CRISPR sensing performance. The combination of multiple crRNA with Cas protein will increase the number of Cas/crRNA binary complexes, leading to activating multiple Cas proteins in the analysis, which will improve the cutting rate of the Cas protein. For example, Fozouni, et al. [28] developed an amplification-free method using three different crRNAs, which increased the minimum detection limit of SRAS-COV-2 by 100 times [28]. Son et al. [29] used several different crRNAs for nucleic acid analysis and greatly improved detection sensitivity [29]. Other amplification free methods, such as highly sensitive electrochemical and optical sensing methods, have also been developed. Li et al established a new biosensor for amplification-free nucleic acid detection via harnessing the trans-cleavage mechanism of Cas13a and ultrasensitive graphene field-effect transistors (gFETs). CRISPR Cas13a-gFET achieves the detection of SARS-CoV-2 down to 1 aM without target preamplification [30-45].

Conclusion

In summary, this paper focuses on the potential application of CRISPR/Cas technology in rapid diagnosis of SARS COV-2, and pre-amplification-based detection methods with amplification-free detection methods on the limit of detection and detection time (Table 2). So far, most CRISPR detection systems, such as SHERLOCK and DETECTR, use preamplification to improve detection sensitivity. However, the preamplification methods complicate the assay design, extend reaction time, and increase testing cost. Recently, digital CRISPR, multiple crRNAs and highly sensitive signal transduction technologies have also begun to be used for direct detection of unamplified samples, which can achieve rapid and highly sensitive detection of SARS COV-2. However, these methods also require high-cost fluorescent or optical detection instruments, microfluidic chips, etc., which limits their wide application. The CRISPR based sensing technology has shown great potential in specific, sensitive, and economical nucleic acid detection, although it is still in its infancy. In view of the limitations of the CRISPR/Cas system itself, it can be predicted that the future CRISPR/Cas technology platform will still rely on the support of other technologies. In addition to the aforementioned PCR and thermostat amplification, more emerging materials and technologies will be combined to the CRISPR/Cas system in the future to develop more practical and intelligent diagnostic tools, which will be suitable for huge applications in the field of POCT (point-of-care testing) and bring new hope for the rapid diagnosis of infectious diseases.

Table 2: Preamplification and amplification-free methods for SARS-CoV-2 detection.

Method	Effector	Amplification Method	Signal Readout	Reaction Time	LOD (aM)	Ref
RPA preamplification	LbCas12a	RPA	fluorescence	40	83	[32]
	AacCas12b	RAA	fluorescence	60	16.6	[33]
	LwaCas13a	RPA	fluorescence	80	16.6	[20]
	LbCas12a	RPA	fluorescence	40	16.6	[34]
	LbCas12a	RPA	colorimetric	50	8.3	[35]
	LbCas12a	RPA	colorimetric	80	1.6	[36]
	LbCas12a	RPA one pot	fluorescence	40	80.3	[18]
	LbCas12a	RPA one pot	fluorescence	60	2	[25]
	LbCas12a	RPA one pot	fluorescence	60	1.5	[37]
	LbCas12a	RPA one pot	fluorescence	60	1.6	[38]

LAMP preamplification	LbCas12a	LAMP	fluorescence	50	26	[39]
	LbCas12a	LAMP	fluorescence	40	16.6	[24]
	LbCas12a	LAMP	fluorescence	35	16.6	[40]
	LbCas12a	LAMP	fluorescence	35	2.5	[41]
	AapCas12b	LAMP one pot	fluorescence	120	23	[42]
	LbCas12a	LAMP one pot	fluorescence	50	8.3	[43]
	AapCas12b	LAMP one pot	fluorescence	60	3.3	[4]
PCR preamplification	AsCas12a	PCR	nanopore	60	22.5	[22]
	LwaCas13a	PCR	fluorescence	52	332	[23]
Digital CRISPR	LbuCas13a	No need	fluorescence	60	30	[44]
	LbuCas13a	No need	fluorescence	15	8.3	[28]
Multiple crRNA	LbuCas13a	No need	fluorescence	120	166	[29]
	LbCas12a	No need	optical	71	83	[45]
Sensitive readout system	LwaCas13a	No need	Electrical	30	1	[31]
	LbCas12a	No need	optical	71	83	[45]

Conflicts of Interest

There are no conflicts of interest to declare.

Acknowledgment

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