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CRISPR-Cas based virus detection: Recent advances and perspectives

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ABSTRACT

Viral infections are one of the most intimidating threats to human beings. One convincing example is the coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2. Rapid, sensitive, specific and field-deployable identification of causal viruses is critical for disease surveillance, control and treatment. The shortcomings of current methods create an impending need for developing novel biosensing platforms. CRISPR-Cas systems, especially CRISPR-Cas12a and CRISPR-Cas13a, characterized by their sensitivity, specificity, high base resolution and programmability upon nucleic acid recognition, have been repurposed for molecular diagnostics, surging a new path forward in biosensing. They, as the core of some robust diagnostic tools, are revolutionizing the way that virus can be detected. This review focuses on recent advances in virus detection with CRISPR-Cas systems especially CRISPR-Cas12a/Cas13a. We started with a short introduction to CRISPR-Cas systems and the properties of Cas12a and Cas13a effectors, and continued with reviewing the current advances of virus detection utilizing CRISPR-Cas systems. The significance and advantages of such methods were then discussed. Finally, the challenges and perspectives were proposed. We tried to provide readers with a concise profile of emerging and fast-expanding CRISPR-Cas based biosensing technology, and highlighted its potential applications in a range of scenarios with regard to virus detection.

1. Introduction

Viruses are small, non-cellular pathogens, which can infect most organisms on the earth (Greber and Bartenschlager, 2017). The infections could seriously threaten human beings, leading to significant social and economic costs. Notable examples of viruses that cause severe infections in recent years include SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus type 2) (Zhu et al., 2020), Zika viruses (Lessler et al., 2016), Ebola viruses (Malvy et al., 2019), influenza A viruses (Gao, 2018), etc. The rapid, sensitive and specific identification of causal viruses is critical to prevent and control the spread of diseases (Benzigar et al., 2020; Morales-Narváez and Dincer, 2020). Current detection methods include antigen-antibody analysis (Amanat et al., 2020; Brangel et al., 2018), polymerase chain reaction (PCR) based techniques (Huang et al., 2018; Smyrlaki et al., 2020), genome sequencing, etc. (Wu et al., 2018). However, some disadvantages do exist within these detection methods, such as cross-reactivity, time-consuming, cost-ineffectiveness as well as expensive instruments and skilled technical staff dependence (Niemz et al., 2011; Pletz et al., 2011). Practically, an ideal approach for pathogen detection should be rapid, sensitive, specific, affordable, sometimes instrument-free and suitable for POCT (Point-of-Care Testing) (Ruiz-Vega et al., 2021).

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated proteins (Cas) constitute a nucleic-acid-based adaptive immune system in bacteria and archaea, which uses RNA-guided nucleases to cleave invading nucleic acids (Garneau et al., 2010; Wiedenheft et al., 2012). Cas13a and Cas12a are CRISPR-Cas class 2 systems and they own collateral nucleic acid

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cleavage activity (also called *trans*-cleavage) after recognizing and cleaving the target sequence (also called *cis*-cleavage) (Liu et al., 2017; Chen et al., 2018). Using this collateral activity, virus derived nucleic acids can be sensitively and specifically detected *in vitro* (Palaz et al., 2021). In this case, CRISPR-Cas13a based SHERLOCK (Gootenberg et al., 2017) and CRISPR-Cas12a based DETECTR (Chen et al., 2018) nucleic acid detection methods have been developed and they come into the spotlight in molecular diagnostics. CRISPR-based diagnostic (CRISPR-Dx) platforms unlock the great potential of CRISPR systems and are poised to solve some long-lasting problems of biosensing such as poor sensitivity and specificity. In this article, we underscored the usefulness and novelty of CRISPR-Cas biosensing by focusing on virus detection. Besides, we provided insights into the potential and emerging applications, the pending challenges as well as the perspectives for futuristic developments of CRISPR-Cas based viral detection, as a fasting expanding subspecialty in biosensing.

2. CRISPR-Cas systems

When it comes to CRISPR, it was firstly discovered in the 1980s and was recognized as the adaptive immune system coupled with Cas proteins against invading foreign DNA and viruses in bacteria and archaea (Garneau et al., 2010; Wiedenheft et al., 2012). According to the organization of effector protein, the systems are divided into two major distinct classes, termed as Class 1 and Class 2, which are further subdivided into different types and subtypes. Class 1 systems include type I, III, and IV, which utilize multi-protein effector complexes (Koonin et al., 2017). Unlike Class 1, Class 2 systems utilize single-protein effectors. According to the effector proteins, Class 2 systems can be further divided into three types and several subtypes, which include type II CRISPR systems such as Cas9, type V systems such as Cas12 (also known as cpf1), and type VI systems such as Cas13 (Makarova et al., 2017). The Class 2 type II nuclease Cas9 remains the most commonly used Cas effector. Through recognizing a short specific sequence, termed as PAM (Protospacer Adjacent Motif), Cas9 recognizes and cleaves the specific double-stranded DNA (dsDNA) under the guidance of a single guide RNA (sgRNA), introducing DNA breaks (Ran et al., 2013). The target dsDNA is cleaved by the RuvC and HNH nuclease domains of Cas9 (O'Connell et al., 2014). CRISPR-Cas9 system has been developed as a powerful genome engineering tool and applied in various fields (Knott and Doudna, 2018; Yin et al., 2018). Also, several other Class 2 systems are subsequently identified and characterized, including type V CRISPR-Cas12a (Zetsche et al., 2015) and type VI CRISPR-Cas13a (Abudayyeh et al., 2016). Cas12a is a programmable RNA-guided DNA nuclease without the requirement of *trans*-activating crRNA (tracrRNA) for crRNA maturation, and it recognizes a T-rich PAM for dsDNA cleavage (Fonfara et al., 2016; Zetsche et al., 2015). Cas13a is an RNA-guided endoribonuclease (endoRNase) that is programmed to recognize and degrade single-stranded RNA (ssRNA) targets carrying complementary sequences (Abudayyeh et al., 2016). Fig. 1 illustrates the schematics of Class 2 CRISPR-Cas systems (Knott and Doudna, 2018). Being different from Cas9, Cas12a and Cas13a intriguingly exhibit collateral, nonspecific activities on random single-strand DNA (ssDNA) or ssRNA respectively upon target recognition (Chen et al., 2018; East-Seletsky et al., 2016). An overview of the features of these three types is listed in Table 1.

Utilizing the collateral activity, Cas13a and Cas12a are respectively explored for nucleic acid detection. In 2017, Feng Zhang's group developed a diagnostic tool named as Specific High-Sensitivity Enzymatic Reporter Unlocking (SHERLOCK) based on CRISPR-Cas13a system (Gootenberg et al., 2017), which allowed ultrasensitive and specific identification of DNA or RNA from clinical samples. Similarly, Jennifer Doudna's group developed a diagnostic approach named as DNA Endonuclease Targeted CRISPR Trans Reporter (DETECTR) based on CRISPR-Cas12a system in 2018 (Chen et al., 2018). Unlike Cas12a, Cas13a requires a relatively simple PAM-like sequence motif termed as protospacer flanking site (PFS), and some Cas13 orthologs, such as LwaCas13a from *Leptotrichia wadei*, do not require PFS, enabling them to target any possible sequence (Abudayyeh et al., 2017). The schemes of SHERLOCK and DETECTR are shown in Fig. 2. Other variants have been soon reported, which provided novel platforms for detecting viruses, pathogenic bacteria and human DNA genotypes, as well as identifying mutations in cancer gene, small molecules, etc. (Li et al., 2019a, 2019b; Peng et al., 2020a, 2020b; Zhou et al., 2020).

3. Virus sensing based on CRISPR-Cas12a/Cas13a systems

CRISPR-Cas12a/Cas13a systems have been leveraged for virus detection, and a range of strategies have been reported (Table S1), which are reviewed and discussed in the following parts.

3.1. Virus sensing with different signal readouts

3.1.1. Virus sensing via fluorescent signals

The first two sensing systems, SHERLOCK and DETECTR, were originally based on the increment of fluorescent intensity. Simply, ssRNA or ssDNA reporter was doubly labeled with a fluorophore (F) at 5′

Table 1
Description and comparison of Cas9, Cas12a, and Cas13a effectors.

Type	II	V	VI
Effector protein	**Cas9**	**Cas12a**	**Cas13a**
Pre-crRNA processing	No	Yes	No
tracrRNA	Yes	No	No
Spacer length	18-24 nt	18-24 nt	22-28 nt
PAM/PFS	3′, G-rich (NGG)	5′, T-rich (TTTN)	3′, non-G-PFS
Substrate	dsDNA	dsDNA, ssDNA	ssRNA only
Cleavage pattern	Blunt	Staggered	Near U or A
Collateral cleavage	No	DNA (ss)	RNA (ss)

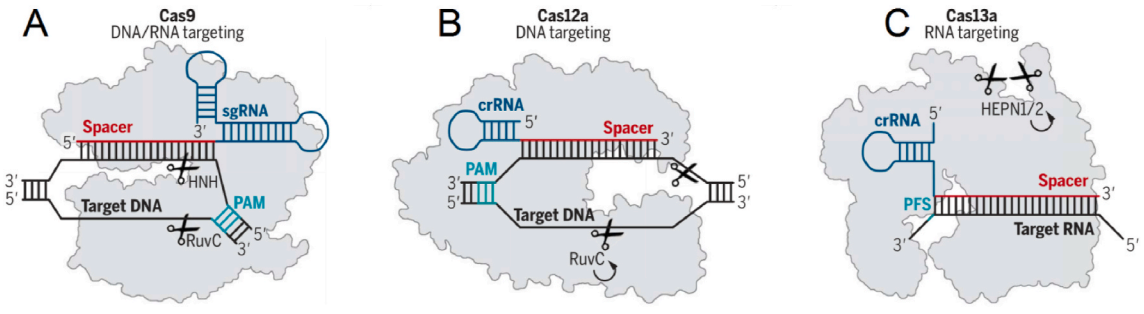

Fig. 1. Schematics of Class 2 CRISPR-Cas systems (Knott and Doudna, 2018). (A) Class 2 type II system: CRISPR-Cas9; (B) Class 2 type V system: CRISPR-Cas12a; (C) Class 2 type VI system: CRISPR-Cas13a. sgRNA is indicated with blue, the spacer sequence is red, PAM or PFS is teal, and single head arrows indicate enzyme activity. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

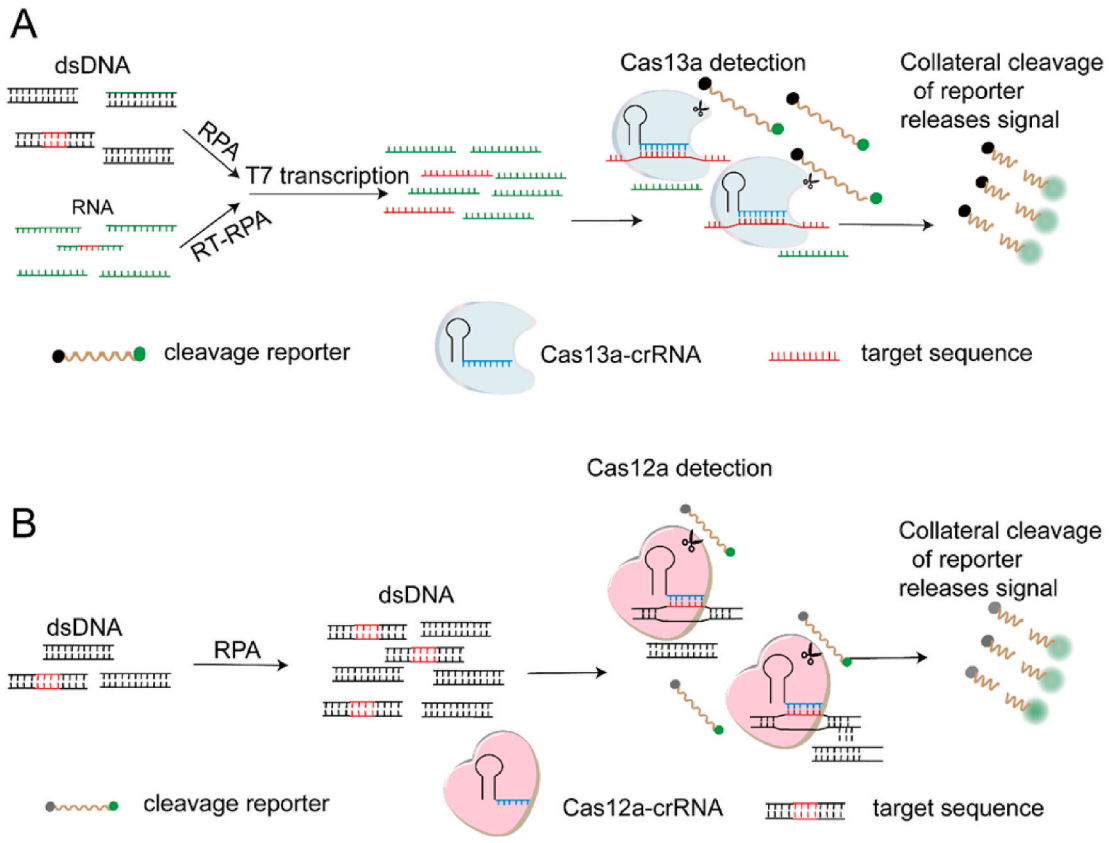

Fig. 2. Schematic of the SHERLOCK and DETECTR. (A) CRISPR-Cas13a based detection (SHERLOCK). Following the RPA (Recombinase Polymerase Amplification) or RT (Reverse Transcription)-RPA, the RNA target recognized by Cas13a-crRNA complex is produced by *in vitro* transcription of the DNA amplicons. On recognition of the RNA target, the *trans*-cleavage activity of Cas13a is activated, resulting in the degradation of the doubly labeled fluorescent reporter. (B) CRISPR-Cas12a based detection (DETECTR). The RPA amplicon is used directly as the target for the Cas12a-crRNA complex. On the recognition of the DNA target, the *trans*-cleavage activity of Cas12a is activated, resulting in the degradation of the doubly labeled fluorescent reporter.

and a quencher (Q) at 3' ends. When the system recognized and bound to the target RNA or DNA, the collateral activity of Cas13a or Cas12a was activated and the ssRNA or ssDNA reporter was cleaved nonspecifically. After the cleavage of ssRNA or ssDNA reporter, the fluorophore was separated from the quencher and the fluorescent signals were recorded for analysis.

SHERLOCK was capable of detecting Zika virus (ZIKV) and dengue virus (DENV), which achieved an astonishing high sensitivity in aM (attomolar, 10^{-18} M) level and single-base resolution (Gootenberg et al., 2017). Moreover, to suit field deployment, Gootenerg et al. also applied a paper-based SHERLOCK termed as SHERLOCKv2 (SHERLOCK version 2) to detect ZIKV RNA with freeze-dried Cas13a. The limit of detection (LOD) of mock ZIKV virus detection on paper-based SHERLOCK was as low as 20 aM, and the sensitivity was slightly reduced (Gootenberg et al., 2018). DETECTR was applied for human papillomavirus (HPV) detection and it was able to differentiate HPV16 and HPV18 by targeting the hypervariable loop V of the L1-encoding gene with specific crRNAs (Chen et al., 2018). In addition, a number of other viruses were also detected using CRISPR-Cas12a/Cas13a based methods, such as canine parvovirus (Khan et al., 2019), avian influenza A viruses (Liu et al., 2019), Ebola and Lassa virus (Barnes et al., 2020), porcine reproductive and respiratory syndrome virus (PRRSV) (Chang et al., 2020), hepatitis B virus (HBV) (Wang et al., 2020a), papillomavirus (Chen et al., 2018), African swine fever (ASF) (Bai et al., 2019), white spot syndrome virus (WSSV) (Chaijarasphong et al., 2019), Japanese encephalitis virus (JEV) (Li et al., 2019a), tobacco curly shoot virus (TCSV) (Smith et al., 2020), Ebola Virus (Qin et al., 2019) as well as SARS-CoV-2 (Broughton et al., 2020; Fozouni et al., 2020; Hou et al., 2020; Patchsung et al., 2020). For SARS-CoV-2 detection, it was reported that near single-copy sensitivity could be achieved by a CRISPR-Cas13a based diagnostic method with fluorescence readouts (Hou et al., 2020). The CRISPR-Cas13a based test could also distinguish the drug-resistance mutation in human immunodeficiency virus (HIV) (Ackerman et al., 2020) and HBV (Wang et al., 2020a) by using specific crRNAs. The sensitivity and specificity of Cas13a/Cas12a mediated virus detection could be further increased by combining Csm6 (Gootenberg et al., 2018), utilizing engineered crRNA (Nguyen et al., 2020), or using multiple crRNAs (Fozouni et al., 2020).

The fluorescence based sensing usually renders ease, lower background, and higher signal-to-noise (S/N) ratio. Apart from microplate reader, a smartphone (Fozouni et al., 2020), LED blue light illuminator (Wang et al., 2020e), portable instrument (Chen et al., 2020a) and microfluidic chip (Qin et al., 2019) were also developed to record the fluorescent signals to amplify the capability for POCT, as shown in Fig. 3A (Ding et al., 2020).

3.1.2. *Virus sensing via colorimetric signals*

Other visualized methods have been developed as well. Gold nanoparticles (AuNPs) based colorimetric assays are advantageous owing to their observable color changes. Aggregation and dispersion behaviors of AuNPs exhibit different distance-dependent optical properties, which can be discerned by naked eye (Zhou et al., 2015). Along with the aggregation of AuNPs, the absorption peak shifts to longer wavelengths and the color of the colloidal solution changes from red to purple, and presents colorless after centrifugation (Liu and Liu, 2017). Utilizing this important feature of AuNPs, Yuan et al. presented a colorimetric gene sensing platform coupled with CRISPR-Cas12a/Cas13a systems (Yuan et al., 2020a). In the platform, a linker ssDNA or RNA was designed, which is capable of hybridization with the AuNPs-DNA probes. When

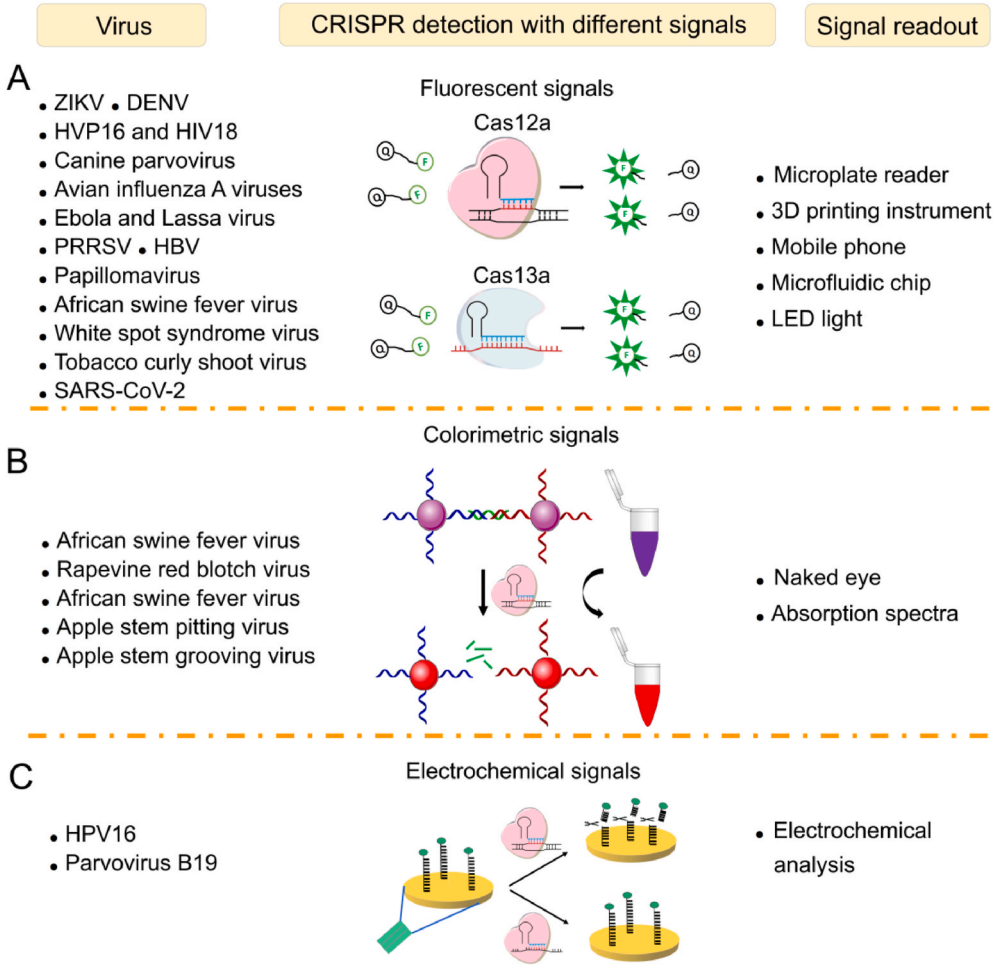

Fig. 3. Virus sensing with different signal readouts. (A) Virus sensing based on CRISPR-Cas12a/Cas13a via fluorescent signals. (B) Virus sensing via colorimetric signals. (C) Virus sensing via electrochemical signals.

the target exists, the *trans*-cleavage activity of Cas12a or Cas13a was activated and it resulted in the degradation of the linker ssDNA or ssRNA, which triggered the dis-aggregation of the AuNPs (Fig. 3B). Utilization of this platform, African swine fever virus was detected by naked eye within 1 h. Combining the *trans*-cleavage activity of Cas12a and DNA functionalized AuNPs, Li et al. generated a plasmonic CRISPR-Cas12a assay for grapevine red blotch virus (GRBV) detection by naked eye (Li et al., 2019d). Hu et al. developed a magnetic pull-down assisted colorimetric diagnosis based on the CRISPR-Cas12a system, termed as M-CDC (Hu et al., 2020). African swine fever virus (ASFV) was successfully detected by M-CDC method with 100 % sensitivity and 100 % specificity. Moreover, a horseradish peroxidase (HRP) enhanced M-CDC was also developed. It could catalyze the TMB to generate a color reaction, achieving the visual detection of pathogens. Colorimetric sensors are not only convenient for visual detection, but also exhibited high sensitivity. In the detection of apple stem pitting virus (ASPV) and apple stem grooving virus (ASGV), the sensitivity of CRISPR-Cas12a based visual assay reached 250 viral copies per reaction, which was comparable to RT-qPCR (Jiao et al., 2020).

3.1.3. *Virus sensing via electrochemical signals*

Electrochemical techniques have drawn significant attention owing to simplicity, low cost and high sensitivity for nucleic acid detection (Drummond et al., 2003; Mozneb et al., 2020). Dai et al. reported an electrochemical biosensing platform based on CRISPR-Cas12a (E-CRISPR), which reached a picomolar sensitivity for human papillomavirus 16 (HPV16) and parvovirus B19 (PB-19) detection (Dai et al., 2019). In this platform, a reporter ssDNA conjugated with a methylene blue (MB) tag and a thiol moiety was designed. The MB tag was for signal transduction and the thiol moiety was for bonding to the sensor surface. When the target existed, Cas12a was activated and cleaved the MB conjugated reporter ssDNA off the sensor surface. Therefore the methylene blue signal transduction was decreased. In contrast, if Cas12a was silenced, the MB conjugated ssDNA was retained on the sensor surface (Fig. 3C). In another study, Xu et al. presented an enhanced electrochemical DNA (E-DNA) sensing platform by combining with CRISPR-Cas12a, which was applied for sensitive and specific detection of the nucleic acid from ssDNA virus and parvovirus B19 (PB-19) (Xu et al., 2020). In the sensor, the conformation of surface signaling probes was changed by the target ssDNA binding, which led to the rate of the electron transfer changing. Combining with CRISPR-Cas collateral activity and the E-DNA sensing platform together, the electrochemical signals were further increased.

3.2. *Virus sensing in POCT via lateral flow assays (LFA)*

Recently, applications of CRISPR-Cas systems in POCT are widely explored, because POCT meets ASSURED criteria: Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end-users (Dai et al., 2020a). Lateral flow assay (LFA) is the technology based on antigen-antibody interaction or target DNA-probe DNA hybridization utilizing conjugated gold, carbon, or colored latex nanoparticles within the conjugate pad (Jiang et al., 2019). The combination of LFA with CRISPR-Cas systems largely expands their utility

especially in POCT owing to the advantages of both CRISPR-Cas and LFA.

The application of the SHERLOCK-LFA assay for instrument-independent detection ZIKV and DENV ssRNA demonstrated that the sample-to-result time was less than 90 min with high sensitivity down to 2 aM (Gootenberg et al., 2018). CRISPR-Cas13a based detection coupled with LFA was also applied for SARS-CoV-2 detection (Patchsung et al., 2020). Patchsung et al. tested 154 clinical COVID-19 samples with the SHERLOCK method (Fig. 4A) (Patchsung et al., 2020). The results showed that 96 % sensitivity was achieved with the fluorescent readout, and 88 % sensitivity was achieved with the lateral flow readout. The specificity of both readouts was 100 %. RNase contamination in the SHERLOCK can obscure testing results. To reduce false negatives or false positives caused by RNase contamination, a multiplexing CRISPR-Cas13a based detection on LFA was developed for both SARS-CoV-2 and RNase contamination detection (Fig. 4B) (Patchsung et al., 2020). Barnes et al. developed CRISPR-Cas13a based diagnostics with both fluorescent readout and lateral flow readout for Ebola virus (EBOV) and Lassa virus (LASV) detection (Fig. 4C) (Barnes et al., 2020). The limit of EBOV synthetic DNA detection of both methods was 10 copies/μL. Moreover, lateral flow readout was combined with the strip reader mobile application (App), enabling to read and analyze the image of test strips by a backend server. Similar to Cas13a based LFA, CRISPR-Cas12a was also fabricated with lateral flow readout for viruses detection, such as African swine fever virus (ASFV) (Wang et al., 2020c), SARS-CoV-2 (Broughton et al., 2020; Guo et al., 2020; Joung et al., 2020; Nguyen et al., 2020), HPV 16 and HPV 18 (Mukama et al., 2020), Epstein-Barr virus (Yuan et al., 2020b) and so on (Fig. 4D). It is necessary to mention that Joung et al. developed a STOPCovid (SHERLOCK testing in one pot, STOP) method, which used a thermostable Cas enzyme AapCas12b for one-pot LAMP/Cas12 detection of SARS-CoV-2 (Joung et al., 2020).

3.3. Multiplexed virus detection

Multiple detection is required in many cases. For example, SARS-CoV-2 infection manifests respiratory symptoms, similar to that of influenza virus. Therefore, it is important to detect two or more pathogens in one test to secure an accurate diagnosis. Utilizing the different cleavage preferences of different Cas proteins on nucleotide sequences, Gootenberg et al. performed a four-channel multiplex detection by using different Cas effectors (Fig. 5A) (Gootenberg et al., 2018). Ackerman et al. developed CARMEN (Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids) platform for multiplex detection of different pathogens (Fig. 5B) (Ackerman et al., 2020). In this platform, CRISPR-Cas13a based detection mixture contained nanolitre droplets that were self-organized and paired with droplets containing amplified samples in a microwell array. Each sample was tested against different crRNAs. Utilizing CARMEN platform, researchers generated a multiplexed detection that was able to simultaneously differentiate 169

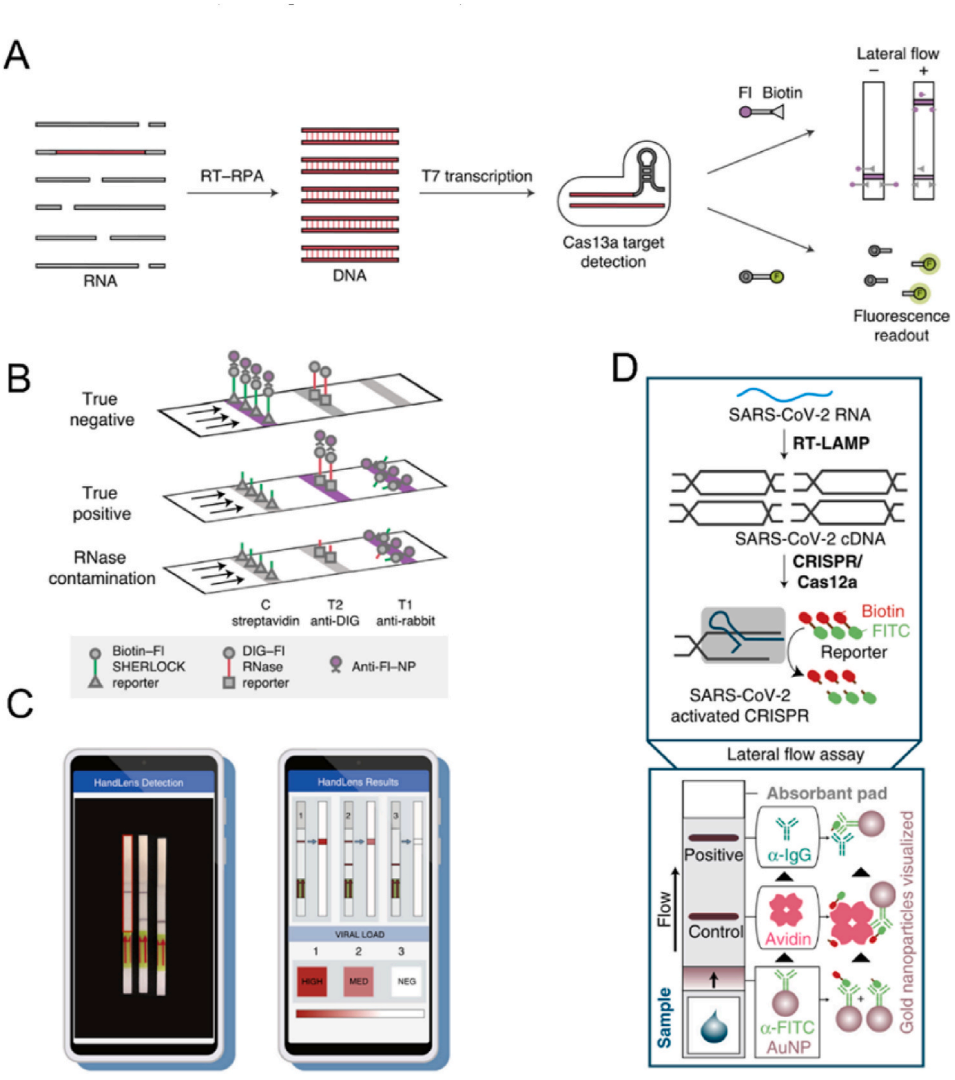

Fig. 4. Virus sensing via paper lateral flow assays. (A) RNA of SARS-CoV-2 detection with CRISPR-Cas13a based lateral flow assay (Patchsung et al., 2020). (B) Paper lateral flow assay for SAR-CoV-2 RNA detection with an internal control to distinguish RNase pollution (Patchsung et al., 2020). (C) SHERLOCK lateral flow strips were quantified by a mobile phone App (HandLens) (Barnes et al., 2020). (D) RNA of SARS-CoV-2 was detected by a CRISPR-Cas12a based lateral flow assay (Nguyen et al., 2020).

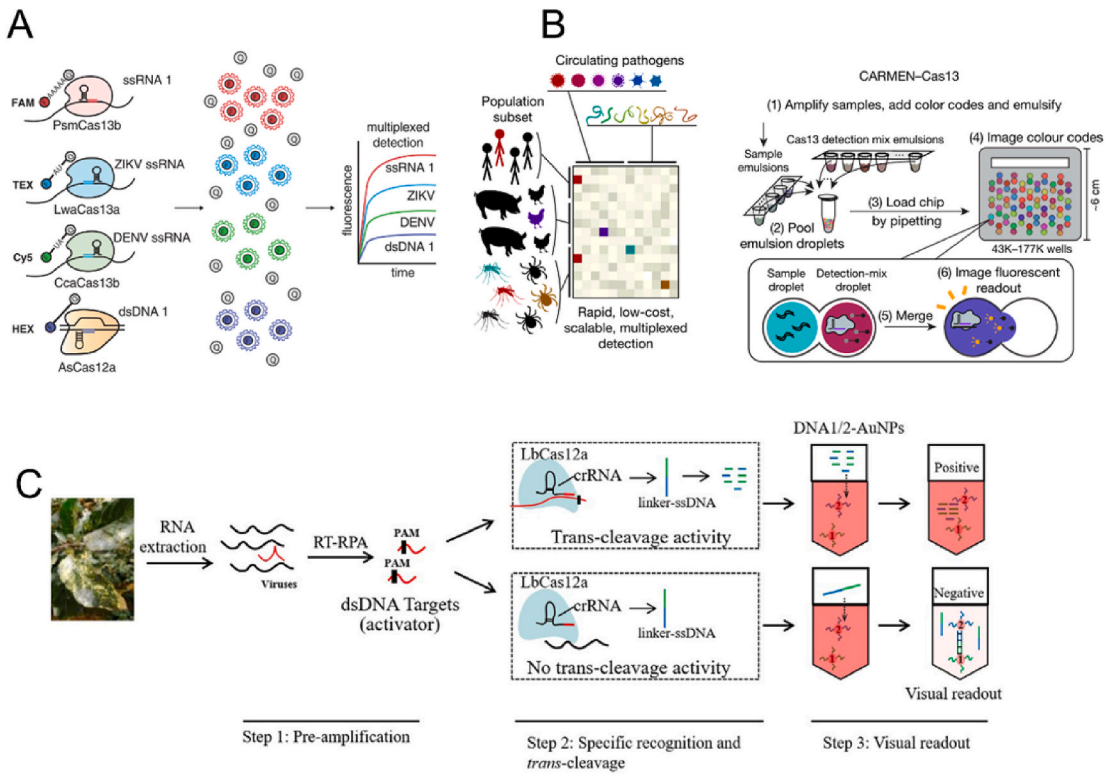

Fig. 5. Multiplexed virus detection with CRISPR-Cas12a/Cas13a. (A) Schematic of multiplexed virus detection with orthogonal collateral activity of CRISPR-Cas12/Cas13 (Gootenberg et al., 2018). (B) The workflow of CARMEN-Cas13a for multiplex identification of circulating pathogens in human and animal populations (Ackerman et al., 2020). (C) Schematic of viral RNA detection with multiplex RT-RPA/Cas12a/AuNPs assay (Jiao et al., 2020).

human-associated viruses and detect SARS-CoV-2 by rapidly incorporating an additional crRNA. CARMEN-Cas13 was also proved to be effective in the identification of several HIV drug-resistance mutations and in subtyping of influenza A strains. Jiao et al. reported a CRISPR-Cas12a based concurrent detection of RNA viruses by using a multiplex RT-RPA and separate LbCas12a/AuNP visual assays (Fig. 5C) (Jiao et al., 2020). Simply, the researchers used five different sets of virus-specific primers for multiplex RT-RPA in a single tube and the amplification products were subsequently tested against different crRNAs. The sensitivity was less than 2.5×10^2 viral copies per reaction for ASPV and ASGV and 2.5×10^3 for apple chlorotic leaf spot virus (ACLSV) and apple scar skin viroid (ASSVd), which was lower than single RT-RPA detection. The efficiency of amplification could be improved by optimizing the parameters, such as primer concentrations, leading to the enhancement of detection sensitivity.

3.4. SNP (Single nucleotide polymorphism) detection

The ability to identify single-nucleotide mutations is crucial in molecular diagnosis and personalized medicine. In terms of virus, single nucleotide variation would sometimes greatly matter in determining subtypes with different infectivity and severity. Gootenberg et al. introduced base mismatches in the target-crRNA duplex to identify the African or American strains of Zika viruses and strain 1 or 3 of Dengue viruses (Gootenberg et al., 2017). Synthetic mismatch crRNAs were able to detect the corresponding strains other than the off-target strains with high sensitivity and specificity, allowing for robust strain identification with a single-base mismatch. HBV and the drug resistance mutations were detected using PCR coupled CRISPR-Cas13a assay (Wang et al., 2020a). By targeting the hypervariable loop V of the L1-encoding gene with specific crRNAs, HPV16 and HPV18 were accurately detected using CRISPR-Cas based lateral flow biosensor (Mukama et al., 2020). Moreover, using ligation strategy and specific Cas13a/crRNA recognition, SARS-CoV-2 D614G mutation was specifically detected (Wang et al., 2021).

3.5. Virus detection by target amplification or amplification-free

To lower the limit of detection, CRISPR biosensing methods are often coupled with nucleic acid amplification. For example, CRISPR-Cas13a based detection coupled with RPA amplification is able to detect extremely low concentrations of ssRNA target down to ~2 aM, much more sensitive than that of Cas13a alone (Gootenberg et al., 2017). Amplification methods usually include conventional PCR and isothermal amplification. Isothermal amplification does not require a thermocycler and can be carried out at one temperature (for example room temperature), which is more convenient for POCT (Giuffrida and Spoto, 2017). The most employed isothermal amplification methods should be RPA and loop-mediated isothermal amplification (LAMP). RPA employs two primers, a single-stranded DNA binding protein (SSB), a recombinase enzyme, and a DNA polymerase for template-directed amplification at ambient temperature (25–42 °C) usually within less than 60 min (Daher et al., 2016). In the study of SARS-CoV-2 detection, RT-RPA was introduced in CRISPR-Cas detection, and the detection sensitivity was as low as 42 RNA copies per reaction (Patchsung et al., 2020). The LAMP utilizes a DNA polymerase to perform DNA synthesis with an auto-cycling strand displacement. LAMP is usually conducted at higher temperatures (60–65 °C) and generates an exponential amplification of the template sequence by utilizing a set of template-specific primers (Notomi et al., 2000; Zhang et al., 2014). Broughton et al. reported that SARS-CoV-2 was detected rapidly (less than 40 min) and sensitively (10 copies per reaction) by CRISPR-Cas12a and RT-LAMP coupled method (Broughton et al., 2020). Also, several methods have been developed for sensitive detection without amplification. Fozouni et al. developed an amplification-free CRISPR-Cas13a assay to directly and quantitatively detect RNA of SARS-CoV-2 extracted from clinical

samples (Fozouni et al., 2020). The assay achieved about 100 copies/μL sensitivity by combining crRNAs that targeted multiple sites of the viral RNA. Nguyen et al. developed a higher sensitive detection platform with engineered CRISPR RNAs (crRNAs). Using the platform, multiple clinically relevant nucleic acid targets were detected and the limit of detection reached femtomolar without any pre-amplification (Nguyen et al., 2020). Besides, Shi et al. designed a CRISPR-Cas12a powered positive feedback circuit and the HBV DNA was detected with attomolar sensitivity (Shi et al., 2021).

3.6. Application of CRISPR-Cas12a/Cas13a for real sample analysis

CRISPR-Cas12a/Cas13a is not only developed for detecting synthesized nucleic acids but also has been applied for virus detection in real samples, such as plasma, serum, urine, swabs, environment and food samples, etc. For infectious samples detection, viral DNA or RNA in the sample was often extracted prior to amplification. For SARS-CoV-2 diagnosis, oropharyngeal and nasopharyngeal swabs were mostly tested. Fozouni et al. directly detected RNA of SARS-CoV-2 from nasal swabs with CRISPR-Cas13a based assay without amplification (Fozouni et al., 2020). In the assay, RNA of SARS-CoV-2 extracted from nasal swabs could be accurately detected under 5 min. Broughton et al. designed a CRISPR-Cas12a coupled lateral flow assay for RNA of SARS-CoV-2 sensitive detection extracted from respiratory swabs (Broughton et al., 2020). To simplify the operation, some studies reported the detection of viral nucleic acid directly from the raw sample matrix without nucleic acid extraction (Barnes et al., 2020; Hu et al., 2020; Myhrvold et al., 2018; Schermer et al., 2020). Myhrvold et al. developed HUDSON (Heating Unextracted Diagnostic Samples to Obliterate Nucleases). This technique enables SHERLOCK to detect virus directly from bodily fluids (plasma, serum, urine), such as Dengue virus from patient samples (Myhrvold et al., 2018). Yuan et al. reported African swine fever virus detection in pig serum samples (Yuan et al., 2020a). In this method, TCEP/EDTA was firstly added into the serum to inactivate the RNase/DNase and heating was conducted to inactivate the virus. It is known that viruses are unimaginably ubiquitous. CRISPR-Cas12a/Cas13a systems were also proved to detect virus found in some other sources (environment, food or animal samples) such as apple leaf, shrimp, canine intestine and animal blood (Chaijarasphong et al., 2019; Jiao et al., 2020; Khan et al., 2019; Sullivan et al., 2019; Wang et al., 2020b). All these samples needed pre-treatment or/and nucleic acid extraction steps depending on each case, but this could be usually done by commercial kits or established methods.

4. Challenges and perspectives

CRISPR-Cas12a/Cas13a based biosensing methods, as the most intensively researched CRISPR-Dx, have advantages such as single-base resolution, at least femtomolar (fM) (without amplification) or attomolar (aM) (with amplification) sensitivity, and instrument-free for some cases. CRISPR-Cas12a/Cas13a based biosensing is able to detect viruses with few copies/reaction and distinguish different subtypes or mutations (Bai et al., 2019; Gootenberg et al., 2017). Additionally, these nucleic acid biosensing systems can be integrated with multiple techniques to satisfy various needs (Ding et al., 2020; Fozouni et al., 2020; Liu et al., 2019; Myhrvold et al., 2018). For example, they can be integrated with paper lateral flow assay, field-effect transistor based biosensor and electrochemical biosensors to realize the on-site diagnostics (Dai et al., 2020a). Moreover, they are suitable for large-scale screening and mass testing for their low cost and the tolerance to various raw samples (Myhrvold et al., 2018; Schermer et al., 2020). Nevertheless, there are still some pending problems, proposed as follows:

4.1. Sequence limitation

CRISPR effector, such as Cas12a, recognizes and cleaves the target dsDNA requiring PAM sequences, which enhances the accuracy of target detection but this, on the other hand, limits the selection of target nucleic acid region. Therefore, fewer choices are left when performing short sequence detection or discriminating SNP, which may restrict the applications. To reduce the dependence on PAM motifs, the PCR products were introduced with PAM sequence by utilizing PAM-containing primers in HOLMES, enabling HOLMES to detect dsDNA in a PAM independent manner (Li et al., 2018). To get rid of the PAM dependence, Ding et al. developed two crRNAs to target nucleoprotein gene of SARS-CoV-2 using CRISPR-Cas12a based assay (Ding et al., 2020). The study showed that dual crRNAs without PAM sequence enabled a higher sensitivity than single crRNA with PAM motif. Similarly, CRISPR-Cas13a mediated target cleavage is limited by protospacer flanking site (PFS), where a non-G base is required. This PFS issue can be easily overcome by selecting the suitable protospacer sequence or applying LwaCas13a effector that is not limited by PFS (Gootenberg et al., 2017).

4.2. Multiplexing and quantification

SHERLOCKv2 has represented the capability for multiple target detection in one single tube by using four different Cas effectors with different cleavage preferences in di-nucleotides (Gootenberg et al., 2018). However, it is severely restricted by the numbers of suitable Cas proteins and the crosscutting among the proteins and reporters may interfere the results of detection. Therefore the discovery of more Cas effectors or the understanding of sequence preference for *trans*-cleavage of Cas12a/Cas13a to allow a more-membered multiplex sensing is urgently needed in this regard. Besides, several other multiplex detection also developed by physical separation, such as CARMEN platform (Ackerman et al., 2020) and the multiplex RT-RPA method (Jiao et al., 2020). However, many works still need to be considered, including the design of a suitable separation structure, primer pairs design, etc. Combining with sensor array or microfluidics, it is potential for CRISPR-Cas to win wide application in multiplex detection (Li et al., 2019c). Bruch et al. designed a CRISPR based electrochemical microfluidic multiplexed biosensor through fabrication of multiple sensing channels (Bruch et al., 2021). For quantitative testing, CRISPR-Cas based assay is difficult to quantify the targets with high concentration, which is easy to reach the signal plateau because of the high sensitivity of the system and the limited reporter molecules (Li et al., 2019a). Additionally, nucleic acid samples are usually subjected to amplification prior to CRISPR-Cas12a/Cas13a processing, the amplification steps would sometimes conceal or distort the true concentrations of original samples, giving rise to misleading results (Kebschull et al., 2015; Sabina and Leamon, 2015). An absolute quantification would usually require an internal standard. The necessity of absolute or relative quantitative detection also needs to be considered in each case, depending on specific purposes.

4.3. Standardization

Standardization is key for the real application of CRISPR-Cas based biosensing, especially in POCT. Reaction buffer, reaction time, reaction composition, temperature, the abundance of reaction inhibitors, DNase/RNase contamination, nucleic acid extraction method, as well as the outputted detection signals, may give rise to variations in the generated results (Broughton et al., 2020; Li et al., 2019a). It may be solved by offering quality control to calibrate the detection. However, the ease of testing would be compromised. Moreover, CRISPR-Cas based biosensing could be potentially used in diverse domains such as food safety, medical diagnosis and plant protection, indicating that bespoke specifications are ideal for practical use and side-by-side comparison. Standardized operations set by official agencies for CRISPR-Cas based biosensing would propel its real use and commercialization in the near future.

4.4. Sample pretreatment

Sample pretreatment is a bottleneck in nucleic acid testing, especially for complexed samples such as food and also for POC applications that are often manually performed. So far, almost all published CRISPR-based biosensors showed that pretreatment of the raw sample is required for subsequent rounds of virus sensing. To ease the sample pretreatment, HUDSON was developed, which amplified the target directly after heating. However, the detection sensitivity was lower than the methods with DNA/RNA extraction, especially for raw samples, such as serum, urine, etc. (Li et al., 2019a; Myhrvold et al., 2018). In the future, developing super Cas effectors that are more robust or biosensing systems with tolerance capability, or establishing sample pretreatment strategies adaptable to biosensing process are greatly needed. More convenient and easy-to-operate instruments could also be customized to suit CRISPR-based biosensing, for example, to facilitate the samples pretreatment and so on.

4.5. Contamination-free and low-background detection

Preventing the cross-reaction and reducing contamination are markedly important for virus detection. Several studies have proposed one-pot detection method that could complete virus detection in one single tube (Chen et al., 2020a; Ding et al., 2020; Pang et al., 2020). However, the detection sensitivity is compromised somehow due to the generation of contaminated nucleic acids, which creates urgent needs for developing contamination-free detection such as high specificity gene amplification technology (Hu et al., 2021). The most feasible strategy for integrating CRISPR-Cas systems with another technology or system is to find a specific nucleic acid molecule as a linker. Because of the high sensitivity nature of CRISPR-Cas systems, it is sometimes inevitable to face a high background signal caused by nonspecific activation of the CRISPR-Cas systems. These nonspecific activation of Cas proteins, could potentially reduce the sensitivity in the CRISPR-Cas integrated biosensing systems (Li et al., 2018).

4.6. Field-deployable detection

CRISPR-Cas based biosensing is a promising alternative to established nucleic acid based diagnostic method that is suitable for the POCT or other field-deployable measurements. A number of strategies have been conducted to promote POC detection; such as sample pretreatment with heating (Myhrvold et al., 2018), isothermal amplification (Gootenberg et al., 2017), different visual readouts (Ali et al., 2020; Jiao et al., 2020) et al. However, these still need multiple operations separately. Simplified operation steps or POC test development may be the solution for complete true field detection. In order to facilitate the application of CRISPR-Cas in POC or field-deployable settings, integration of CRISPR-Cas with other platforms such as LFA, microfluidics, biochip, nanotechnology, mobile phone technology, cloud computing and artificial intelligence will be an effective solution (Berg et al., 2015; Ibrahim et al., 2020; Quesada-González and Merkoçi, 2017; Weiss et al., 2020). Microfluidics has provided a powerful tool for the development of point-of-care detection. Combining the CRISPR-Cas12a/Cas13a with microfluidic devices, the detection methods are compatible with the characteristics of rapidity, high sensitivity, low cost, automation and on-site. While microfluidics also brings about new challenges, such as the possible cross-reactivity by diffusion (Bruch et al., 2021), the requirements of appropriately designed channel volume for scale-up (Ramachandran et al., 2020), off-chip manual steps such as sample lysis, nucleic acid extraction as well as nucleic acid amplification (Ramachandran et al., 2020). To solve some of those challenges, futuristic work could be directed towards the development of fully automated microfluidic assays via integration process (Chen et al., 2020b). For instances, CRISPR-Cas12a reagents could be pre-loaded on reaction chamber of the microfluidics, and lyophilized at -80 °C using freeze-drying system in a multiplexed, CRISPR-based microfluidic detection of SARS-CoV-2 (Yin et al., 2021).

In conclusion, viral infections and outbreaks seriously impact human health as well as global economy. In view of this, rapid, sensitive, specific and field-deployable detection is instrumental to effectively control and prevent the spread of diseases. CRISPR-Cas systems, as excellent gene editing tools, are also developed as novel and powerful diagnostic techniques (Abudayyeh and Gootenberg, 2021). For example, CRISPR-Cas9 has been utilized to detect viruses through linking Cas9 as a recognition element and expanded its capability by other designs (Azhar et al., 2021; Dai et al., 2020b; Jiao et al., 2021; Lin et al., 2021; Pardee et al., 2016; Wang et al., 2020d). Different from CRISPR-Cas9, CRISPR-Cas12 and CRISPR-Cas13 are developed as powered diagnostic tools with their *trans*-cleavage activity, which have ushered a new era in molecular diagnostics. To date, lots of different viruses have been detected by SHERLOCK, DETECTR or the variants in the laboratory, including SARS-CoV-2. These virus detection methods usually achieve fM to aM concentration sensitivity and single-base resolution. Different reporting and data collecting methods have been developed, such as fluorescent readouts, colorimetric readouts, electrochemical readouts, etc. POCT through CRISPR-Cas12a/Cas13a based lateral flow assays has been realized. Besides, multiplex detection has also been tested using different Cas effectors or multiple physical separations. To ease the virus detection in raw samples, some pretreatment methods, such as HUDSON, have been developed to directly amplify and detect the virus without sacrificing the sensitivity. Another advantage of the CRISPR12a/Cas13a based detection method is their cost-effectiveness. As the CRISPR-Cas12a/Cas13a based detection are usually cheaper and the detection does not require expensive instruments. A typical SHERLOCK paper test costs $0.61 per test, which is generally less than other detection methods (such as real-time PCR) (Aman et al., 2020; Gootenberg et al., 2017). In spite of the advantages, some challenges are still remained to be solved. In summary, CRISPR-Cas based biosensing represents a conceptually novel paradigm for virus detection. More importantly, it is fast expanding in its development and revolutionizing the way that viruses can be detected by combing with some existing methods to meet diverse needs. It can be conceived that the CRISPR-Cas technology could be readily put into the toolkit of analysts in a “plug in” manner. CRISPR-Cas systems have enormous yet unexploited potentials in diagnostics. We believe that multidisciplinary integration, such as materials, engineering, cloud computing and artificial intelligence would make these systems unprecedentedly powerful in biosensing in the coming future.

CRediT author contribution statement

Lijuan Yin: Writing-original draft, Writing-review & editing. Shuli Man: Conceptualization, Project Administration. Shengying Ye: Project Administration. Guozhen Liu: Writing-review & editing. Long Ma: Conceptualization, Investigation, Supervision, Project Administration, Writing-original draft, Writing-review & editing, Funding acquisition.

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

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