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REVIEW ARTICLE

Recent Advances in CRISPR-Based Biosensors for Point-of-Care Pathogen Detection

Noor Mohammad,[1,2] Shrinivas Siddhivinayak Katkam,[1] and Qingshan Wei[1,*]

Abstract

Infectious pathogens are pressing concerns due to their heavy toll on global health and socioeconomic infrastructure. Rapid, sensitive, and specific pathogen detection methods are needed more than ever to control disease spreading. The fast evolution of clustered regularly interspaced short palindromic repeats (CRISPR)-based diagnostics (CRISPR-Dx) has opened a new horizon in the field of molecular diagnostics. This review highlights recent efforts in configuring CRISPR technology as an efficient diagnostic tool for pathogen detection. It starts with a brief introduction of different CRISPR-Cas effectors and their working principles for disease diagnosis. It then focuses on the evolution of laboratory-based CRISPR technology toward a potential point-of-care test, including the development of new signaling mechanisms, elimination of preamplification and sample pretreatment steps, and miniaturization of CRISPR reactions on digital assay chips and lateral flow devices. In addition, promising examples of CRISPR-Dx for pathogen detection in various real samples, such as blood, saliva, nasal swab, plant, and food samples, are highlighted. Finally, the challenges and perspectives of future development of CRISPR-Dx for infectious disease monitoring are discussed.

Introduction

Infectious pathogens such as viruses and bacteria have been a constant threat to human beings. Even though the development of antibiotics has plummeted the mortality rate caused by bacterial infections,[1] viral pathogens are continuously ravaging our social and economic life. COVID-19 is the stark proof of it.[2] Apart from the severe acute respiratory syndrome-coronavirus type-2 (SARS-CoV-2, the causing pathogen of the ongoing COVID-19 pandemic), Zika viruses (ZIKV),[3] influenza A viruses,[4] and Ebola viruses (EBOV)[5] are constantly infecting people. Viral and bacterial infections are not only a threat to humans/animals, but also pose a severe threat to plants.[6,7] Therefore, it is a dire necessity to detect the specific infection rapidly and sensitively.[8]

Current approaches for pathogen detection are mainly based on molecular assays, including antigen–antibody-based immunoassay (e.g., enzyme-linked immunosorbent assay),[9] polymerase chain reaction (PCR),[10,11] and DNA sequencing.[12] However, these conventional detection methods are time-consuming, dependent on expensive instruments, and require skilled technical staff. The complex procedure, field-portability, and high labor cost are the inherent issues of existing molecular detection methods.[13] Alternatively, a simple, cost-effective, rapid, sensitive, and field-deployable platform is desired for detecting pathogens at the point of care (POC). A POC test (POCT) can be defined as diagnostic tests administered outside the central laboratory at or near the location of the patient.[14] Based on the complexity of the test and difference in application sites, it can refer to rapid tests performed in remote or battlefield clinics, or self-testing units frequently used at home, agricultural fields, or other resource-limited sites such as outer space stations.[15]

While there are different requriments for a POCT to be used in a specific application, a general POCT should follow the ''ASSURED'' criteria suggested by the WHO, including affordable, sensitive, specific, user friendly, rapid/robust, equipment free, and delivered.[16] Among those, sensitivity and specificity are still the most

[1]Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, North Carolina, USA; [2]Department of Chemical Engineering, Bangladesh University of Engineering and Technology, Dhaka, Bangladesh.

*Address correspondence to: Qingshan Wei, Department of Chemical and Biomolecular Engineering, North Carolina State University, 911 Partners Way, Campus Box 7905, Raleigh, NC 27695, USA, E-mail: qwei3@ncsu.edu

challenging features to address on a low-cost and miniaturized platform.

Clustered regularly interspaced short palindromic repeats (CRISPR) initially emerged as a cutting-edge genome-editing tool. Together with Cas proteins (CRISPR-associated protein), the CRISPR-Cas system acts as the adaptive immune system in bacteria and archaea, using RNA-guided nucleases to cleave the invading viral DNA/RNA.[17,18] Among various CRISPR systems, Cas12a (aka. Cpf1) and Cas13a (aka. C2c2) are widely studied that show collateral nucleic acid cleavage activity (or *trans*-cleavage) after recognizing and cleaving the target nucleic acid sequence (or *cis*-cleavage).[19,20] This feature of Cas enzymes makes CRISPR a powerful diagnostic tool for nucleic acid detection with exquisite sensitivity and selectivity.[21]

Since the introduction of the Specific High-Sensitivity Enzymatic Reporter un-LOCKing (SHERLOCK) platform[21] and DNA Endonuclease-Targeted CRISPR *Trans* Reporter (DETECTR) platform,[20] many new CRISPR-based diagnostic (CRISPR-Dx) platforms have been demonstrated, which unlock the great potential of CRISPR technology for solving the long-desired goal of achieving highly sensitive and specific nucleic acid detection (e.g., pathogen detection through target genes) through a relatively simple procedure.[22–34]

While the majority of the current research focuses on the biology and diagnostic potential of the CRISPR technology in laboratory settings, the transformation of laboratory-based CRISPR-Dx into POCT is also emerging.[35–38] Promising examples of POC CRISPR tests have been highlighted in several recent reviews.[39,40] In this study, instead of reviewing more POC CRISPR examples based on different Cas effectors or detection methods, we summarize several ongoing and most promising efforts to facilitate the bench-to-bedside translation of the CRISPR technology, including simplifying the CRISPR assay procedure, simplifying the CRISPR signal readout format, miniaturizing the CRISPR reaction, and enabling multiplexed detection at the POC (Fig. 1). We illustrate such process by using CRISPR-based pathogen detection as an example.

The review article starts with a brief overview of different CRISPR-Cas effectors and their potential for disease diagnostics. Then, the emerging trends of transforming CRISPR-Dx into POC methods are discussed, such as the development of preamplification-free CRISPR reaction and digital CRISPR (digiCRISPR) assay. It is then followed by a discussion of the applications of CRISPR-Dx for POC pathogen detection from different types of raw samples, including real human, plant, and food samples. Finally, the challenges and perspectives of the future development of POC CRISPR-Dx for infectious disease monitoring are discussed.

Brief Overview of CRISPR Technology

CRISPR-Cas systems are nucleic acid-based adaptive defense mechanisms in the archaea and bacteria that protect these microorganisms from invading viruses.[41,42] The system was initially adopted by researchers for genome editing and therapy. Its potential as a molecular diagnostic tool has been amplified through the course of the COVID pandemic, where a single-guide RNA (sgRNA) is designed to target any desired sequences and the trans-cleavage activity of Cas effectors is utilized to generate the signals. This system is highly

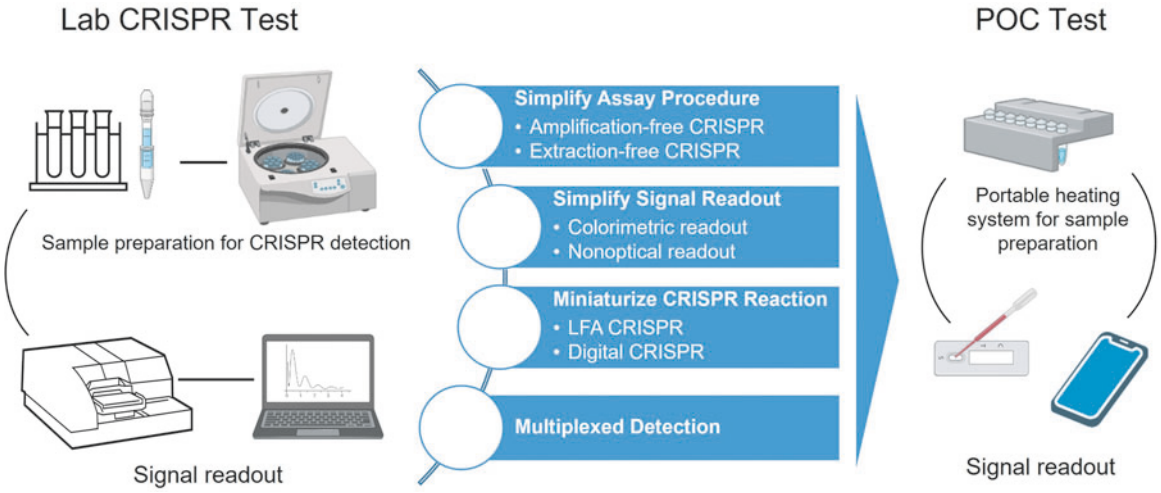

FIG. 1. Illustration of key efforts transforming laboratory-based CRISPR-Dx into POC tests. CRISPR, clustered regularly interspaced short palindromic repeats; CRISPR-Dx, CRISPR-based diagnostic; POC, point of care.

programmable, allowing it for easy adaption to different targets with high specificity and sensitivity, which makes it a promising tool for nucleic acid detection.[43]

Many CRISPR-Cas systems work on the same principle, but they come in a wide range of structures and functions. Indeed, the enormous number of extant prokaryote viruses leads to a wide range of antiviral mechanisms, implying that both the CRISPR loci and the Cas protein can change quickly, resulting in a wide range of variations. As a result, both the CRISPR array and the Cas enzymes are quite unique.[43] Various CRISPR-Cas systems used for pathogen detection are discussed here. Table 1 summarizes some key features of representative Cas proteins, such as the Cas9, Cas12, and Cas13 enzymes.

CRISPR-Cas 9

In the Cas9 system, to form a complex of tracrRNA and crRNA with Cas9, a noncoding tracrRNA (75–110 bp) is responsible for creating a pre-crRNA. After that, a non-Cas RNase III ribonuclease cleaves the tracrRNA:pre-crRNA to produce a ready-to-use tracrRNA:crRNA for the Cas9 interference complex.[44] The Cas9 uses this tracrRNA:crRNA guide to find a specific protospacer adjacent motif (PAM) sequence (i.e., 5′-NGG-3′) in the nontarget DNA strand and forms the guide RNA-target duplex 10–12 nts away from PAM to disfavor the reannealing of dsDNA duplex.[45] The cleavage then occurs in the presence of metallic ions, which produces a blunt break at 3 bp upstream of the PAM.[43,44,46] So far, several pathogens such as the Zika virus,[47] *Listeria monocytogenes*,[48,49] and African swine fever virus (ASFV)[49] have been detected utilizing the cleavage mechanism of CRISPR-Cas9 system.

CRISPR-Cas12

The Cas12 is a class 2 type V Cas enzyme with many subtypes such as Cas12a, Cas12b, Cas12c, and Cas12f (previously known as Cas14), as given in Makarova *et al.*[50] Among these subtypes, Cas12a (Cpf1) is the most studied. They are capable of self-processing the crRNA into their nuclease domains without the help of RNA or any additional proteins. It can self-process a pre-RNA by cutting it just before the stem-loop, resulting in a mature crRNA guide that can be used to build an interference complex.

A conformational shift occurs when the crRNA: Cas12a complex is formed, similar to the fit-induced mechanism in Cas9.[44] The crRNA: Cas12a recognizes a T-rich PAM (5′-TTTN-3′) in the nontarget DNA strand and cleaves this strand at 8–19 bases from the 3′ end of the PAM. Then, the opposite strand (target strand) of the DNA is cut at 23 bases from the PAM, giving a 5′ sticky overhang.[44,51] Also, Cas12a from certain bacteria (e.g., *Lactobacillus bacterium*) has been discovered to have a nonspecific ssDNA collateral activity (*trans*-activity). The creation of biosensing systems has been sparked by the discovery of *trans*-cleaving activity induced by *cis* activity. The Cas12 recognizes its nucleic acid target and triggers *trans*-activity, which can be amplified and reported using different readout strategies such as the fluorescent, colorimetric, and electrochemical methods.[44] Cas12a is among the most explored effectors for disease diagnosis so far.

For example, DETECTR is one of the pioneering CRISPR-Cas12-based virus detection platforms. After the discovery of DETECTR, many CRISPR-Cas12-based sensing platforms have been developed for detecting a variety of pathogens, such as ASFV,[52,53] white spot syndrome virus (WSSV) in shrimp,[54] human papillomavirus 16 (HPV16) and human papillomavirus 18 (HPV18),[55] Epstein–Barr virus (EBV),[56] and SARS-CoV-2.[24,30,57–59]

CRISPR-Cas13

Cas13 is an RNA-guided protein of class 2 type VI of Cas enzymes with RNAse activity.[41,42] Once activated by the target RNA, it cleaves adjacent RNAs through the *trans*-activity.[44] All known type VI systems act without tracrRNA. After the crRNA:Cas13 complex is formed, two HEPN domains in the NUC lobe are activated, and they are responsible for both *cis*- and *trans*-activity. The activity of Cas13 enzymes is based on the presence of protospacer flanking site (PFS), which is analogous to PAMs for DNA targets. A PFS at the 3′-protospacer strand is required for the Cas13a enzymes.[60] Cas13b enzymes, on the contrary, require a PFS in each protospacer strand. However,

Table 1. Characteristics of Cas9, Cas12, and Cas13 proteins[44]

Name of effector protein	*Cas9*	*Cas12*	*Cas13*
Type	II	V	VI
tracrRNA	Required	Not required	Not required
Spacer length, nt	18–24	18–24	22–28
PAM/PFS	3′ G-rich (NGG)	3′ T-rich (TTTN)	3′ non-G-PFS
Active site	Two domains (HNH and RuvC)	Single domain (RuvC)	Two domains HEPN
Target	dsDNA	dsDNA/ssDNA	ssRNA
Collateral cleavage	No	ssDNA	ssRNA

PAM, protospacer adjacent motif; PFS, protospacer flanking site.

Cas13a from *Leptotrichia wadei* (LwaCas13a) is routinely used to bypass the PFS requirement.[21]

CRISPR-Cas13 *trans* cleaves the RNA substrate once it recognizes and *cis* cleaves the target sequence. In the SHERLOCK system, the target RNA is detected by coupling CRISPR reaction with a reverse transcriptase–recombinase polymerase amplification (RT-RPA) isothermal amplification step. Cas13 cleaves the surrounding RNA transcripts and RNA reporters when Cas13 binds the target RNA sequences.[21] After developing SHERLOCK, many CRISPR-Cas13-based platforms have come into light for the detection of a wide range of pathogens. For example, canine parvovirus type II,[61] porcine reproductive and respiratory syndrome virus (PRRSV),[62] EBOV, lassa virus (LASV) cases,[23] avian influenza A (H7N9) virus,[63] SARS-CoV-2,[22,28,29,64–67] and various pathogenic bacteria[68] have been detected by the CRISPR-Cas13-based mechanism in the last few years. Because of its potential for multiplexing and high-throughput screening, Cas13-based diagnostics has become a promising tool for rapid pathogen detection.

Among three categories, Cas12 and Cas13 brought a significant breakthrough in CRISPR-Dx due to their unique *trans*-cleavage activity, which greatly simplifies signal generation. In contrast, the Cas9 system still requires a complex guide RNA design and signaling mechanism, and that is why Cas9 is no longer the preferred option for CRISPR diagnostics.

Evolution of CRISPR-Dx: From Laboratory to POC

CRISPR-based pathogen detection technology has evolved rapidly in the past few years after its first introduction. Many different signal readout mechanisms and sensor configurations have been demonstrated. This section first briefly summarizes the fundamentals of a conventional laboratory-based CRISPR-Dx test and then moves onto the recent trend of CRISPR-Dx toward POC applications such as preamplification-free CRISPR and digiCRISPR.

The early demonstration of CRISPR technology for pathogen detection started with the detection of ZIKV with CRISPR-Cas9.[47] The tool came into the real spotlight after the discovery of the *trans*-cleavage mechanism of CRISPR-Cas12[20] and CRISPR-Cas13.[21] A typical CRISPR-based detection process involves several steps, including the extraction of nucleic acids, preamplification of the target genes, and detection through a fluorescence signaling mechanism. Nowadays, CRISPR-Dx has evolved over a short period into many different detection platforms. For instance, some research groups utilized colorimetric readout instead of fluorescence signaling, and some others developed CRISPR-based sensing platforms based on nonoptical (e.g., electrochemical signaling) signaling mechanisms. In addition, various platforms have been developed to bypass the nucleic acid extraction or preamplification steps. Besides, CRISPR-Dx has also evolved into a multiplexed platform for detecting a panel of targets at a time.

Traditional CRISPR-Dx

CRISPR-based detection mechanism started with a few studies using Cas9 effector.

For example, Pardee *et al.* used Cas9 enzyme in their ''toehold switch sensor'' for detecting ZIKV. The toehold switch sensor involves a hairpin structure that blocks lacZ from translation. The blockage is relieved upon the presence of complementary trigger RNA (i.e., transcribed RNA from a target DNA). Consequently, the LacZ enzyme is translated, which changes the yellow substrate (chlorophenol red-β-D-galactopyranoside) on a paper disc into a purple product (chlorophenol red). In the presence of the target, dsDNA was generated by a nucleic acid sequence-based amplification reaction. Then, the CRISPR-Cas9 cleaved dsDNA. As a result, the RNA transcribed from the cleaved DNA cannot activate the toehold switch sensor due to the lack of sensor trigger sequence. In this way, the Cas9 was used in combination with an oligonucleotide colorimetric sensor for the detection of the target. This system was used in detecting and differentiating two different ZIKV genotypes with a single-base resolution.[47]

CRISPR-based detection becomes broadly attractive after the discovery of the *trans*-cleavage mechanism of Cas12 and Cas13 enzymes. Cas12a and Cas13a are widely studied CRISPR-Cas systems that show *trans*-cleavage activity after recognizing and *cis*-cleaving the target sequence.[19,20] This *trans*-cleavage mechanism has been widely utilized for developing CRISPR-based biosensors.[69]

Traditional CRISPR-based detection involves preamplification (e.g., RPA, RT-RPA, loop-mediated isothermal amplification [LAMP], or reverse transcriptase–LAMP [RT-LAMP]) of extracted target nucleic acid, and then detection of amplified targets by the *trans*-cleavage of fluorescent reporter molecules by the Cas enzymes. A typical fluorescent signaling mechanism involves an ssRNA or ssDNA reporter labeled with a fluorophore molecule (F) at the 5′ end and a quencher molecule (Q) at the 3′ end. When Q is at the proximity of F, fluorescence is quenched due to the Forster resonance energy transfer (FRET) mechanism. Upon the addition of target nucleic acid (DNA or RNA), the gRNA directs the Cas enzyme (Cas12 for DNA target; Cas13 for RNA target) to recognize the target. The binding with the complementary target sequence triggers the Cas enzyme to cleave the fluorescent reporters nonspecifically. The

trans-cleavage breaks the FRET pair, and a recovered fluorescence signal is achieved for analysis.[20,21,70]

In 2017, Gootenberg *et al.* developed a CRISPR-Cas13-based SHERLOCK platform, which detected RNA targets with attomolar sensitivity and single-base mismatch specificity. This platform involved an RPA/RT-RPA preamplification step and utilized the degradation of nonspecific RNA reporters by Cas13a enzymes to generate signals. The authors successfully detected specific strains of ZIKV and dengue virus (DENV), and distinguished pathogenic bacteria with their SHERLOCK platform.[21] Furthermore, the same research group developed a paper-based SHERLOCK (SHERLOCK version 2) to detect ZIKV RNA with freeze-dried Cas13a to make the CRISPR-Dx more suited for field deployment.[71]

Chen *et al.* developed the CRISPR-Cas12a based DETECTR platform, which detected both ssDNA and dsDNA. They detected HPV with attomolar sensitivity, and the system was able to distinguish HPV16 and HPV18 by targeting the hypervariable loop V of the L1-encoding gene.[20] The mechanism of traditional CRISPR-based fluorescent detection is illustrated in Figure 2A. Later, many other pathogens were detected by using a similar fluorescent signaling mechanism. For example, H7N9 viruses,[63] canine parvovirus,[61] ASFV,[52] and SARS-CoV-2[22,28–30,57,67] were detected successfully with fluorescent CRISPR assays.

CRISPR Detection Without the Preamplification Step

Traditional CRISPR-Dx faces a few challenges, especially toward POC applications. For instance, these techniques require preamplification steps such as RPA, RT-RPA, LAMP, or RT-LAMP to achieve the desired detection sensitivity (e.g., attomolar or femtomolar concentration of targets). Since the preamplification step complicates and lengthens the overall detection procedure, it is appealing to develop simpler CRISPR-Dx, which excludes this step. CRISPR-Dx platforms without preamplifications are better suited for POC detection. Several platforms have been developed for sensitive detection without preamplification. For example, Nguyen *et al.* developed a femtomolar-level detection platform with engineered crRNAs. Prudent design of crRNAs enhanced the collateral cleavage activity of the CRISPR-Cas12 enzyme as high as 3.5-fold compared with the wild-type crRNA. It also improved the specificity for target recognition significantly.[72]

Shi *et al.* proposed a CRISPR-Cas-only amplification network (CONAN) to detect nucleic acids without preamplification while maintaining a great sensitivity. They designed the switchable-caged guide RNA (scgRNA) to construct a positive feedback circuit based on CRISPR-Cas12a. This assay was capable of detecting hepatitis B virus (HBV) with attomolar sensitivity.[73] In this study, we need lots of optimization to design the scgRNA to be thermodynamically and thermally stable for the CONAN platform.

Fozouni *et al.* constructed an amplification-free CRISPR-Cas13a assay to quantitatively detect RNA of SARS-CoV-2 extracted from clinical samples by combining several crRNAs that targeted multiple sites of the viral genome. This assay achieved about 100 copies/μL sensitivity within 30 min and detected pre-extracted RNA from a set of positive clinical samples in less than 5 min. Although this platform showed an impressive turnaround time, its procedure of finding several appropriate crRNAs was tedious.[66] This tedious procedure is about to end with a very recent discovery of machine learning-based Activity-informed Design with All-inclusive Patrolling of Targets (ADAPT), which helps design multiple crRNAs efficiently and swiftly.[74]

Lately, Liu *et al.* developed a new sensing mechanism termed fast integrated nuclease detection in tandem (FIND-IT) that detected SARS-CoV-2 from patients' samples (respiratory swap samples) without target RNA preamplification. In this assay, Cas13a and Csm6 were combined in a single pot reaction. Eight crRNAs were designed for Cas13a to target eight locations of the SARS-CoV-2 genome. After the activation of Cas13a, it cleaved an activator oligonucleotide, which further activated Csm6. The Csm6 then cleaved F-Q reporters and produced detectable fluorescence signals. This preamplification-free FIND-IT approach demonstrated an limit of detection (LOD) of around 30 molecules/μL in 20 min.[28]

In this study, all of the diagnostic platforms provided an impressive sensitivity, but none of them used the raw samples directly for detection. Rather, almost all of them need to first extract the nucleic acid targets from the raw samples. Still, by bypassing the preamplification step, pathogen detection with the CRISPR-Cas system is becoming simpler and better suited for POC testing.

CRISPR Detection Without the Nucleic Acid Extraction Step

For pathogen detection, viral DNA or RNA often needed to be extracted from the raw sample matrix before CRISPR reaction. However, this sample preparation step makes the overall detection assay laborious and time-consuming. It is also one of the major hurdles in converting CRISPR-Dx into a POCT. Several techniques have been reported to evade the nucleic acid isolation step and detect the pathogens directly from the raw sample

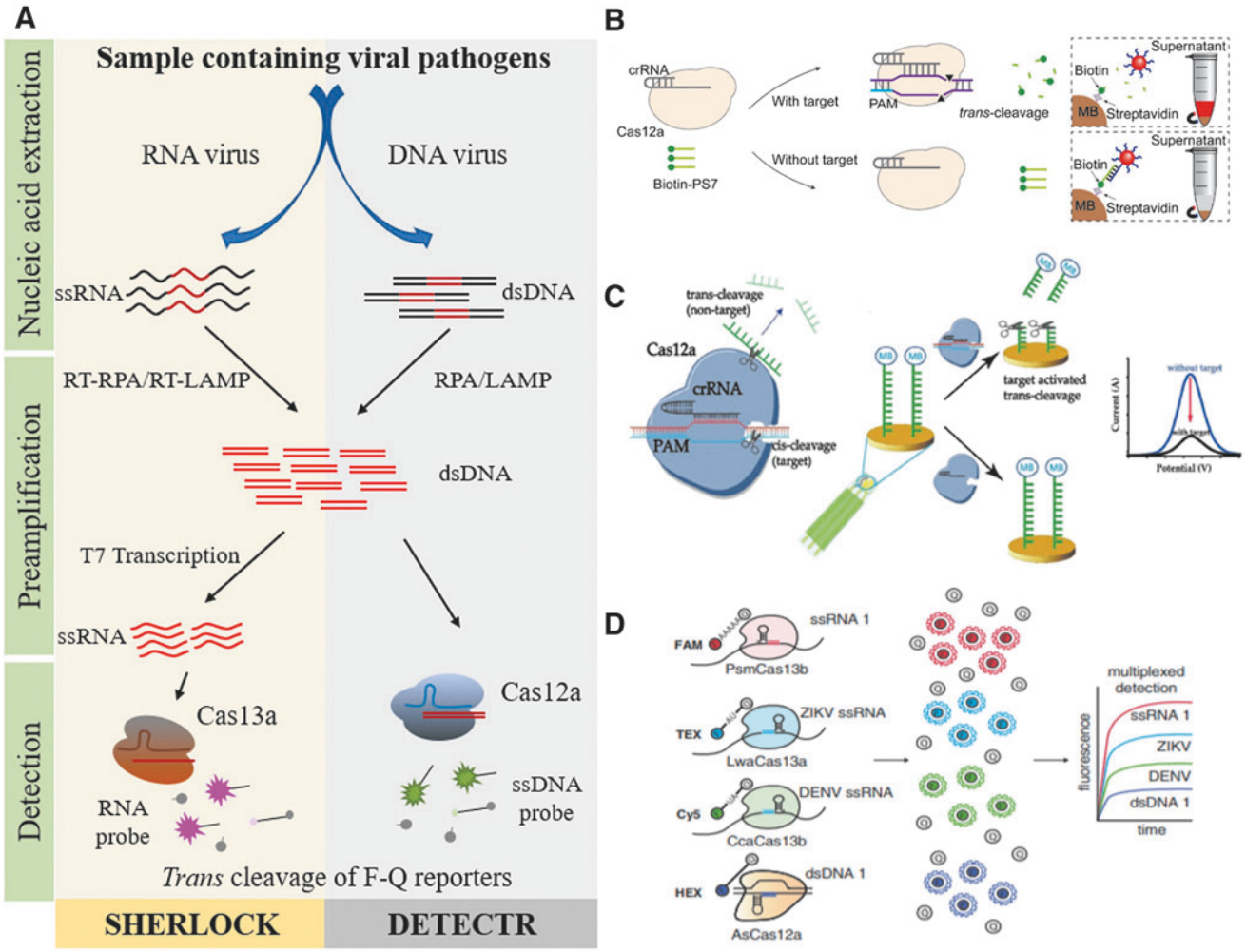

FIG. 2. CRISPR-Dx technologies. **(A)** Traditional CRISPR-Dx platform (SHERLOCK and DETECTR); **(B)** CRISPR-Cas12a-based colorimetric detection utilizing gold nanoparticles as color reporters[79]; **(C)** CRISPR-Cas12a-based detection utilizing electrochemical signaling mechanism[84]; **(D)** CRISPR-Cas12a- and CRISPR-Cas13-based multiplex detection using different fluorophore dyes.[71] ASFV, African swine fever virus; crRNA, CRISPR RNA; DENV, dengue virus; DETECTR, DNA Endonuclease-Targeted CRISPR *Trans* Reporter; LAMP, loop-mediated isothermal amplification; MB, magnetic microbead; MB (in *blue color*), methylene blue; NTS, nontarget strand; PAM, protospacer adjacent motif; RPA, recombinase polymerase amplification; RT-LAMP, reverse transcriptase–LAMP; RT-RPA, reverse transcriptase–recombinase polymerase amplification; SHERLOCK, Specific High-Sensitivity Enzymatic Reporter un-LOCKing; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA; TS, target strand; ZIKV, Zika virus.

matrix without nucleic acid extraction. For instance, Myhrvold *et al.* developed the Heating Unextracted Diagnostic Samples to Obliterate Nucleases (HUDSON) technique that enables SHERLOCK to detect viruses directly from plasma, serum, and urine samples. This technique enabled DENV detection directly from patient samples in <2 h without using any sophisticated instrument.[75] The HUDSON technique made the CRISPR technology suitable for detecting pathogens from a wide range of clinical samples.

Utilizing a similar technique, Yuan *et al.* detected ASFV in pig serum samples. In this method, the authors first added tris (2-carboxyethyl) phosphine hydrochloride (TCEP)–ethylenediaminetetraacetic acid (EDTA) into the serum to inactivate the DNase/RNase followed by heating to inactivate the virus.[76] Arizti-Sanz *et al.* constructed a sensing platform named streamlined highlighting of infections to navigate epidemics (SHINE) by combining HUDSON and SHERLOCK. This platform detected SARS-CoV-2 from unextracted samples

(nasopharyngeal patient samples) within 50 min with 100% specificity and 90% sensitivity when compared with reverse transcription quantitative real-time PCR (RT-qPCR).[67]

Recently, the same research group developed SHINEv2, which utilized the FastAmp lysis reagent instead of heating steps and used lyophilized reagents for detecting SARS-CoV-2 in home settings. This assay demonstrated 50-fold greater sensitivity than conventional antigen-based tests and 100% specificity when tested for 96 patient samples. In addition, SHINEv2 succeeded in differentiating the Alpha, Beta, Gamma, and Delta variants of SARS-CoV-2.[77]

Colorimetric CRISPR Detection

Colorimetric signaling mechanism for CRISPR-Dx is also popular due to its simple signal readout (e.g., naked eye or smartphone) and potential to be configurated in a lateral flow assay (LFA) format, which is better suited for POC detection. The recent development of nanotechnology has greatly expanded colorimetric assays based on nanoparticle dispersion and aggregation behavior.[78]

For example, the color of the colloidal gold nanoparticle (AuNP) solution changes from red to purple due to the redshift of absorption peak upon the aggregation of AuNPs.[19] Utilizing these optical properties of AuNPs, Hu *et al.* built a magnetic-assisted colorimetric diagnosis based on the CRISPR-Cas12a (M-CDC) platform to detect ASFV.[79] Upon target recognition, the CRISPR-Cas12a system triggered *trans*-cleavage activity and degraded biotinylated ssDNA substrates (Biotin-PS7). The degradation of Biotin-PS7 resulted in a decrease in the magnetic pull-down of DNA-AuNP probes. Thus, the supernatant remained colorful even after the separation of streptavidin-coated magnetic beads with an external magnet.

This M-CDC assay platform detected ASFV from infected pig serum samples with 100% sensitivity and 100% specificity (Fig. 2B).[79] Yuan *et al.* proposed a CRISPR-Cas12a- and CRISPR-Cas13a-based colorimetric sensing platform. They used a linker ssDNA or RNA, which could hybridize with the AuNP-conjugated DNA probes. Upon the activation of Cas12a or Cas13a system, the linker oligos were degraded, resulting in the disaggregation of AuNPs. This colorimetric sensing platform was able to detect ASFV by the naked eye in an hour.[76] This platform and the previous M-CDC platform simplified the DNA extraction step with a simple chemical/heat treatment process. Yet, both platforms required nucleic acid preamplifications steps, which could potentially limit the application of these platforms to POC detection.

A few studies have been reported for detecting plant diseases with colorimetric CRISPR-Dx. For instance, utilizing the *trans*-cleavage activity of Cas12a and red shifting property of AuNPs upon aggregation, a plasmonic CRISPR-Cas12a assay was developed by Li *et al.* They detected grapevine red blotch virus (GRBV) using their novel platform with the naked eye.[6] Jiao *et al.* applied a similar concept and detected apple stem grooving virus (ASGV) and apple stem pitting virus (ASPV) from raw infected leaf samples. This platform provided comparable sensitivity (LOD = 250 viral copies per reaction) to RT-qPCR.[7] These CRISPR-based plant disease detection methods required a tedious nucleic acid extraction step. This tedious step could be replaced with an alternative rapid microneedle-based nucleic acid extraction procedure.[80] However, these studies overall suggest that CRISPR-based colorimetric detection not only facilitates visual detection but also maintains excellent detection sensitivity.

CRISPR Detection with Nonoptical Readout

Newer CRISPR-Dx systems not only utilize optical readout (e.g., fluorescence-based, colorimetric), but also have evolved to nonoptical formats such as an electrochemical signal acquisition. The popularity of electrochemical signaling mechanism is increasing because it is simple, inexpensive, and sensitive for pathogenic nucleic acid detection.[81,82] Several electrochemical biosensing platforms based on CRISPR-Cas12a (e.g., E-CRISPR) have been reported so far. Hajian *et al.* combined the gene targeting capacity of CRISPR-Cas9 with graphene field-effect transistor (gFET) to construct a CRISPR-Chip. This CRISPR-Chip comprised a three-terminal gFET which utilized graphene as a channel between source and drain electrodes. The graphene was functionalized with Cas9/sgRNA. Upon the presence of a target, hybridization between the target and sgRNA occurred, which modulated the electrical characteristics of gFET resulting in an electrical signal. This nonoptical signaling output was enshrined for detecting infectious diseases with a low copy number.[83]

Dai *et al.* proposed an E-CRISPR platform that incorporates a reporter ssDNA conjugated with a methylene blue tag and a thiol molecule (Fig. 2C). Methylene blue was used for signal transduction, and thiol moiety was attached with the gold-plated electrode. In the presence of target DNA, CRISPR-Cas12a was activated, and *trans*-cleaved the methylene blue-tagged ssDNA reporter. It then decreased the current flow to the electrode surface. Using this E-CRISPR platform, they detected HPV16 and parvovirus B19 (PB-19) at picomolar sensitivity.[84]

Another electrochemical biosensor was constructed by Xu *et al.* They developed an enhanced electrochemical DNA (E-DNA) sensing mechanism using CRISPR-Cas12a,

which helped achieve a femtomolar detection limit without enzymatic amplification. CRISPR-enhanced E-DNA sensor comprised a thiolated hairpin signaling strand. The thiolated end of the hairpin was bound to the gold electrode, and the other end was tagged with methylene blue. In the presence of target, the hairpin opened, which led to an increased electron tunneling distance. Again due to the *trans*-cleaving activity of CRISPR-Cas12a, the electrochemical signaling probe from the electrode surface was released completely. In this study, a decrease of current due to two sequential mechanisms (conformation induced change and CRISPR enhanced change) helped detect ssDNA virus and PB-19 with excellent sensitivity and specificity.[85]

To implement E-CRISPR for POC settings, Bruch *et al.* integrated microfluidic chips with an electrochemical readout system for detecting brain tumor marker miRNA-19b in patient serum samples. The chip contained an electrochemical cell, channels, and a streptavidin-immobilized area to capture 6FAM-ssRNA-biotin (reporter of Cas13a). The reaction mixture contained the glucose oxidase-labeled antifluorescein antibody and glucose. In the presence of targets, *trans*-activated Cas13a cleaved the reporter molecule and released 6FAM from the immobilized area. The assay readout was based on the production of H_2O_2 from glucose by the labeled glucose oxidase. This assay platform detected miRNA-19b down to 10 picomolar in buffered solutions without the preamplification step.[86] Although no POC applications of E-CRISPR assay have been demonstrated yet, this research shows the strong POC potential, such as chip miniaturization and simple readout.

Digital CRISPR

DigiCRISPR is a relatively new development in CRISPR-Dx to miniaturize the assay format, improves the detection sensitivity, and skips the preamplification step. The digital assay involves microfluidic microwells for converting the bulk solution-based analog CRISPR assay into nanoliter reactions to run thousands of discrete single-molecule CRISPR assays on a chip. The wells/spots containing the target generate fluorescence signals from that well (positive well). On the contrary, no signals will be detected from the wells/spots with no target (negative well). Through counting the number of positive or negative wells, analyte concentration can be attained by Poisson distribution fitting.

For example, Ding *et al.* constructed CRISPR-Cas12a-based digital warm-start CRISPR (dWS-CRISPR) assay for sensitive and quantitative detection of SARS-CoV-2 in clinical samples. dWS-CRISPR involved the partitioning of bulk samples into subnanoliter aliquots within a QuantStudio 3D digital chip. Reverse transcription–dual-priming isothermal amplification and CRISPR-Cas12a reactions were performed in each well/spot in the chip. This sensing platform detected SARS-CoV-2 down to 5 copies/μL in the QuantStudio digital chip (Fig. 3A).[87] We have incorporated smartphones for the first time for quantifying a digital Cas12a assay for sensitive DNA detection in the POC settings. This handheld system detected HBV and HPV DNA markers with fM sensitivity without any preamplification (Fig. 3B).[26] CRISPR-Cas12a based RApid DIgital Crispr Approach (RADICA) was developed by Wu *et al.* for detecting EBV from human B cells and patients' serum.[27]

Digitization-enhanced CRISPR-Cas-assisted one-pot virus detection (deCOViD) was constructed by Park *et al.* for detecting SARS-CoV-2 RNA virus from clinical samples.[25] DigiCRISPR increases the sensitivity and accelerates the conversion of laboratory-based CRISPR assay into a microfluidic chip-based inexpensive POCT since it may replace sophisticated readout devices with a simple smartphone.

Lateral Flow CRISPR

Numerous studies have been reported for pathogen sensing through LFA at POC. LFA has been used for virus detection based on target DNA-probe DNA hybridization, which utilizes conjugated gold or other colored nanomaterials within a conjugate pad as color reporters.[88] The incorporation of LFA with CRISPR reaction helps expand the conventional antibody–antigen-based LFA system to a programmable and more specific nucleic acid testing platform. For instance, Gootenberg *et al.* combined LFA with the SHERLOCK platform for detecting ZIKV and DENV. They detected the target molecule in less than 1.5 h with an LOD of 2 attomolar without any sophisticated instruments.[71] Utilizing a similar type of platform, Patchsung *et al.* detected SARS-CoV-2[22] from 154 clinical COVID-19 samples with 88% sensitivity and 100% specificity in an LFA readout (Fig. 3C).

Barnes *et al.* detected EBOV and LASV with an LOD of 10 copies/μL in the LFA readout (Fig. 3D).[23] Similar to CRISPR-Cas13a-based LFA, CRISPR-Cas12a-LFA was also constructed for detecting various pathogens, such as ASFV,[53] HPV16 and HPV18,[55] EBV,[56] and SARS-CoV-2.[57,72,89]

Together, the recent development of digiCRISPR and CRISPR-LFA has shown the great potential of transforming laboratory CRISPR reactions into rapid, user-friendly, sensitive, specific, and field-deployable POCTs.

Multiplexed CRISPR Detection

Detection or screening of multiple targets/pathogens from the sample is a frequent requirement in many

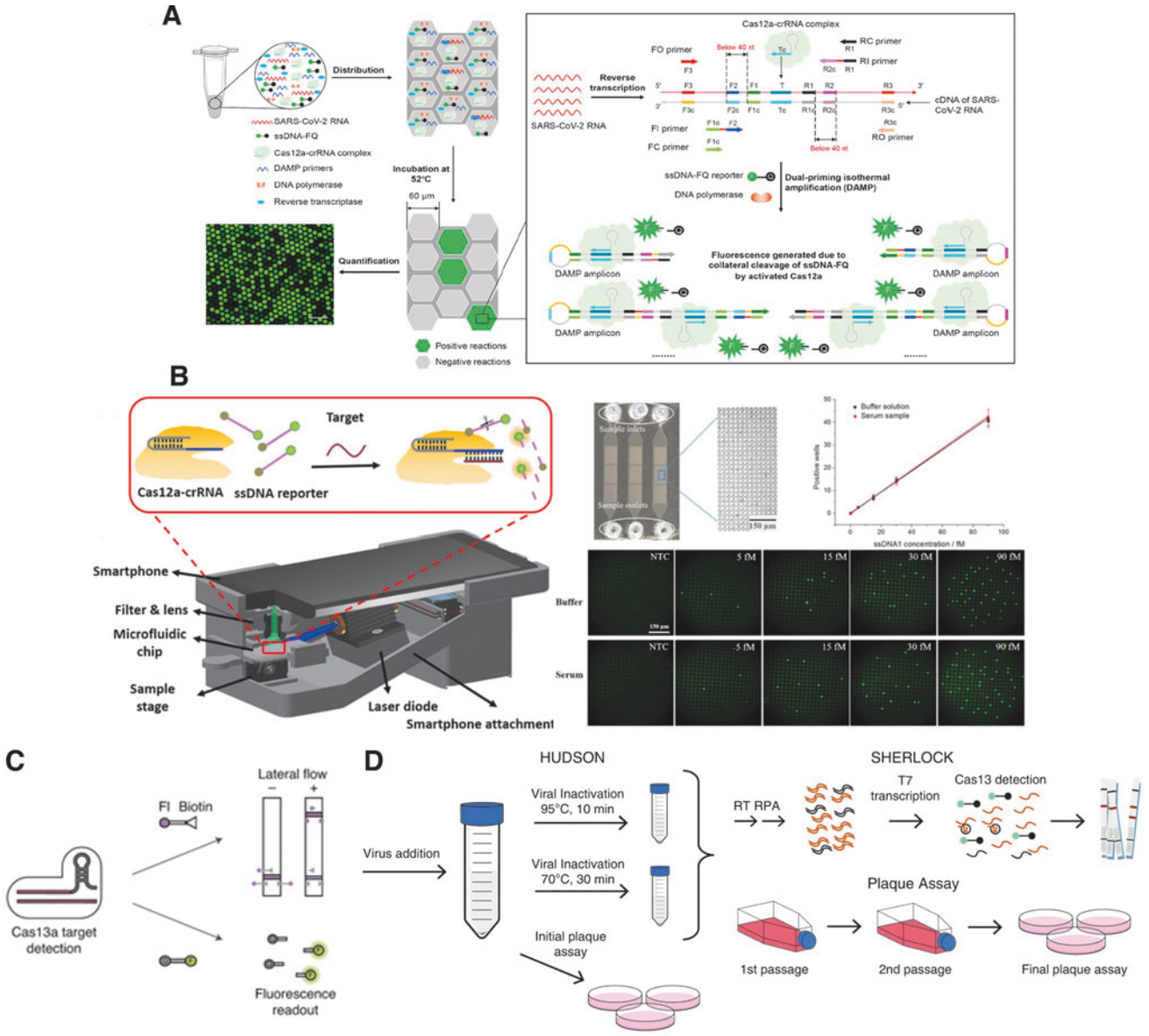

FIG. 3. DigiCRISPR and POC diagnostics. **(A)** SARS-CoV-2 detection using digiCRISPR platform[87]; **(B)** cell phone digiCRISPR for sensitive detection[26]; **(C)** SHERLOCK-based POC detection in LFA[22]; **(D)** SHERLOCK coupled with HUDSON for detecting pathogen detection from raw samples in LFA.[23] digiCRISPR, digital CRISPR; FC, forward competitive; FI, forward inner; FI, fluorescein; FO, forward outer; HUDSON, Heating Unextracted Diagnostic Samples to Obliterate Nucleases; LFA, lateral flow assay; NTC, no-target control; RC, reverse competitive; RI, reverse inner; RO, reverse outer; SARS-CoV-2, severe acute respiratory syndrome-coronavirus type-2.

cases. For example, chikungunya infection manifests similar symptoms to DENV; SARS-CoV-2 infection is similar to the influenza virus infection. Methods that can differentiate and identify specific pathogens are thus needed by multiplexed analysis of multiple targets at a time. Although POC multiplex CRISPR detection is yet to be demonstrated, some exciting progress on multiplexed CRISPR-Dx in laboratory settings is worth highlighting. Many multiplexing concepts expect to be implemented in the POCT soon in the future.

For instance, Gootenberg *et al.* utilized the different cleavage preferences of Cas proteins on nucleotide sequences to develop a four-plex detection system by using four different Cas effectors (i.e., PsmCas13b, Lwa-Cas13a, CcaCas13b, and AsCas12a) (Fig. 2D).[71] Accordingly, they formulated four F-Q reporters with four

different dye molecules and four different types of nucleotide sequences (e.g., 5′FAM/AAAAA/Q3′, 5′TEX/AU/Q3′, 5′Cy5/UA/Q3′, 5′HEX/NNNNN/Q3′) for detecting four different targets simultaneously in a single tube. Using this method, they successfully detected ssRNA1, ZIKV, DENV, and ssDNA1 in a single reaction.

Ackerman *et al.* developed a CRISPR-Cas13a-based Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids (CARMEN) platform for the multiplexed detection of different pathogens. They detected 169 human-associated viruses by creating nanolitre droplets containing amplified samples in microfluidic chips and then tested them against different crRNAs.[64] In this study, the benefit of multiplexing was achieved by compartmentalizing the original samples into different microreactions.

Later, Welch *et al.* developed microfluidic CARMEN (mCARMEN) and used commercially available Fluidigm microfluidics. mCARMEN required less sample and provided a faster and less labor-intensive alternative to CARMEN. mCAMREN detected six SARS-CoV-2 variants, including the Delta and Omicron.[90] Jiao *et al.* constructed a CRISPR-Cas12a-based RNA plant virus detection platform using a multiplex RT-RPA. This research group separated the amplified samples into five tubes and detected five different disease markers.[7]

POC CRISPR-Powered Pathogen Detection from Real-Life Samples

Pathogen detection from various real-life samples such as human, animal, plant, and food samples is of great interest. However, working with real-life samples is not a straightforward task. Higher patient-to-patient variability, the presence of potential inhibitors, and complex sample matrices may significantly interfere assay performance and therefore result in lower detection sensitivity and specificity. In addition, target nucleic acids often need to be extracted from real-life samples before they can be added into the CRISPR reaction. Those nucleic acid extraction and purification steps are currently done by using a range of DNA/RNA extraction kits in the laboratory that still require complex equipment (e.g., centrifuge) and professional personnel. Yet, CRISPR-Dx evolved significantly in recent years to help detect pathogens from human, animal, plant, and food samples. A few representative examples, which are demonstrated/potentially applicable for POC field level detection, are highlighted here.

Pathogen Detection from Human/Animal Samples

Most CRISPR-based pathogen detections have been reported by analyzing various human or animal samples comprising blood/serum samples, cell/tissue samples, saliva samples, and swab samples.

Blood/serum samples

Several studies have been reported for detecting pathogens from human blood or serum samples. Barnes *et al.* constructed CRISPR-Cas13a-based diagnostics targeting EBOV, namely the SHERLOCK-EBOV assay platform. They developed a user-friendly protocol and incorporated mobile phones for reporting the results to facilitate their sensors to be used on site. They detected 16 clinical samples taken from suspected EBOV disease patients in Sierra Leone with 100% sensitivity and 100% specificity during the 2014–2016 West Africa outbreak.[23] Animal blood/serum samples from the infected animal are also a vital source of pathogens.

Hu *et al.* built an M-CDC platform to detect ASFV from pig serum samples. The M-CDC platform involved HUDSON-like protocol for sample pretreatment, RPA for preamplification, and CRISPR-Cas12a system for target detection. The M-CDC assay platform helped detect ASFV-infected pig serum samples with 100% sensitivity and 100% specificity. In addition, they developed horseradish peroxidase-enhanced test strips based on LFA and detected synthetic target, which is superior to the traditional test strip in terms of simplicity, specificity, and portability.[79] Utilizing a similar technique, Yuan *et al.* detected ASFV in pig serum samples. They added TCEP–EDTA into the serum to inactivate the DNase/RNase, followed by heating to inactivate the virus.[76] Hu *et al.* and Yuan *et al.* did not directly show the detection of pathogens in the POC setting, yet their effort to simplify the real-life sample pretreatment process is a milestone in converting the CRISPR-Dx platforms into POC detection.[76,79]

Cells/tissue samples

Cells/tissue specimens from infected humans or animals can be used to diagnose diseases. Wang *et al.* developed a CRISPR-Cas12a-based lateral flow detection (CRISPR-Cas12a-LFD) for detecting ASFV. For on-site detection, unlike extracting the genomic DNA, they used a room temperature sample lysis kit and rapidly detected ASFV from the clinical samples in the field (total time $\sim$1 h).[53] Mukama *et al.* built a high-fidelity CRISPR-Cas12a-based lateral flow biosensor to detect HPV16 and HPV14 from cervical exfoliated cell samples. This lateral flow biosensor detected HPV16/HPV18 from 21 clinically obtained exfoliated cell samples (7 positives and 14 negatives) with 100% specificity. The process was fast with an inexpensive AuNP-based readout, suitable for on-site pathogen detection efficiently and specifically.[55] Pathogens from animal cells/tissues were also detected by CRISPR-Dx.

For example, Chang *et al.* developed a CRISPR-Cas13a-based sensitive and specific diagnostic platform for detecting PRRSV. After receiving 37 tissue

samples collected from different farms, viral RNA was extracted with a standard protocol, amplified with RPA, and finally detected by the CRISPR-Cas13a reaction. Thirty-two samples were found positive among 37 samples, and these results agreed with the results from RT-qPCR. This group showed that the enhanced Cas13a could detect PRRSV from clinical tissue samples in an LFA with 100% specificity, which validates its clinical application in the field without expensive/bulky equipment.[62]

Saliva samples

Similar to other bodily fluids such as blood or serum, saliva from an infected person/animal can also contain different nucleic acid biomarkers. CRISPR-Dx has been used to detect these nucleci acid biomarkers for disease diagnosis purposes. For instances, a SHERLOCK platform was utilized for detecting ZIKV in saliva samples by Myhrvold *et al*. They incorporated the HUDSON mechanism with SHERLOCK to pretreat the raw sample (Fig. 4B). A simplified pretreatment process and colorimetric readout with lateral flow strips make this technique field deployable.[75] de Puig *et al.* constructed minimally instrumented SHERLOCK (miSHERLOCK) for detecting SARS-CoV-2 from saliva samples. They utilized battery-powered incubation and cell phone-based detection to run miSHERLOCK. This platform detected RNA targets down to 1 copy/μL, which matched the LOD provided by the U.S. Centers for Disease Control and Prevention.[29]

We previously incorporated RT-RPA and CRISPR-Cas12a to detect SARS-CoV-2 from patients' saliva

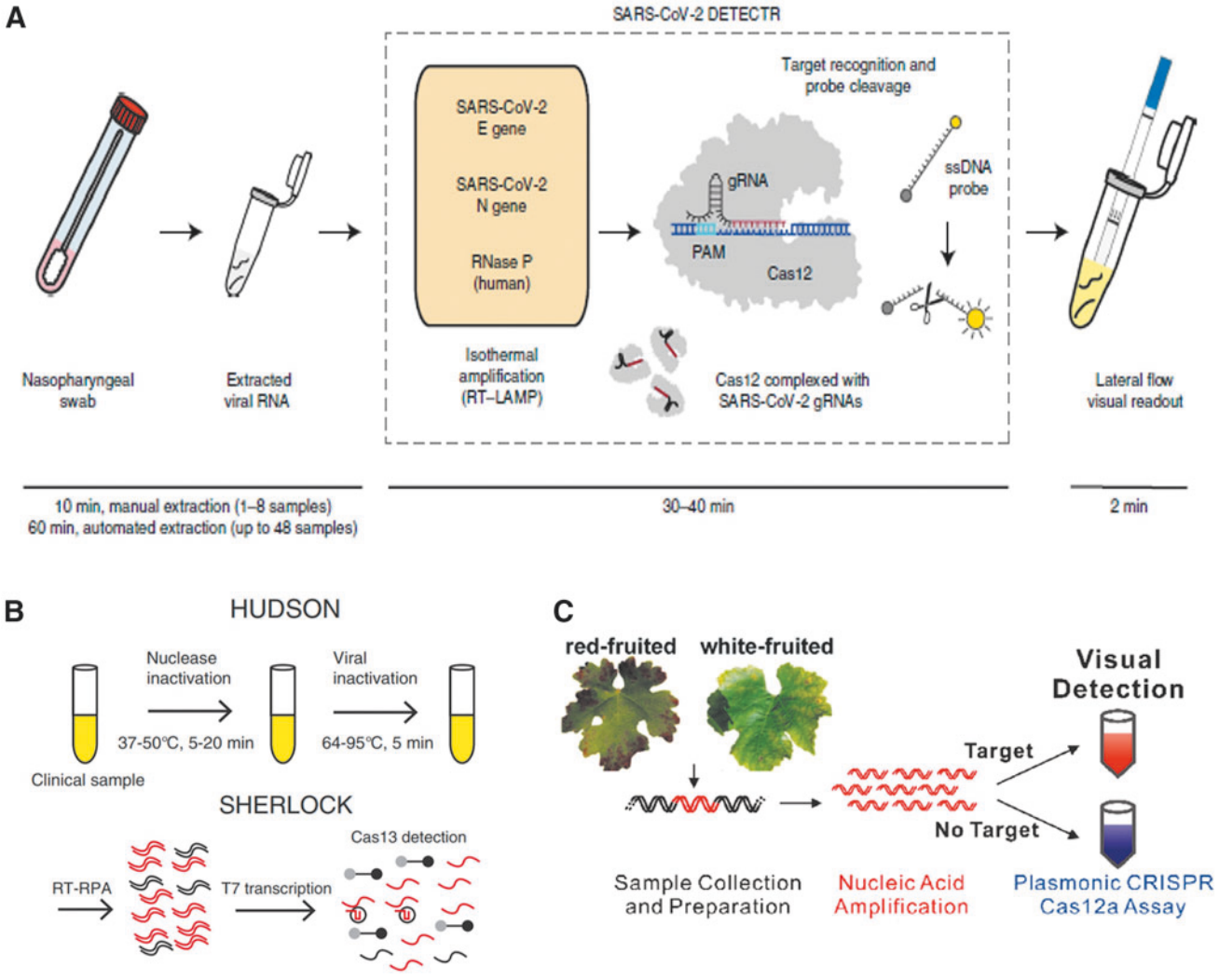

FIG. 4. CRISPR-based pathogen detection from real-life samples. **(A)** SARS-CoV-2 detection from nasopharyngeal swab[57]; **(B)** ZIKV detection from patients' saliva samples[75]; **(C)** GRBV detection from grapevine leaves.[6] GRBV, grapevine red blotch virus; gRNA, guide RNA.

samples using a smartphone. This POC diagnostic system was used to detect SARS-CoV-2 from infected nonhuman primates' saliva, and the results showed that the viral load in oropharyngeal samples was 3.6- to 124-fold higher than that in the nasal swab.[30] Both de Puig *et al.* and our group utilized smartphones for CRISPR signal readout, a trend to make the CRISPR system better suited for POC detection.

Swab samples

Pathogen detection from nasal/nasopharyngeal swabs (e.g., SARS-CoV-2) is also a common disease screening method and well adapted by the public during the outbreak of COVID-19. Fozouni *et al.* designed a Cas13a-based assay that utilized multiple crRNAs to detect and quantify SARS-CoV-2 RNA from patient samples with 100% accuracy without a preamplification step. They used a mobile microscope to quantify the assay signals, which makes the sensing platform suited for low-cost POC detection.[66]

Liu *et al.* applied their FIND-IT concept for sensitive detection of SARS-CoV-2 from patients' respiratory swap samples. They detected around 30 molecules/μL of targets in 20 min without preamplification. To convert FIND-IT into a POC testing platform, they performed the assay in a microfluidic chip with reaction chambers, a heating element (37°C), and a compact fluorescence imaging system.[28] FIND-IT excluded the preamplification step and miniaturized the whole detection module, which is well suited for POC detection. On the contrary, it still required a nucleic acid extraction step before the assay reaction.

Patchsung *et al.* examined SARS-CoV-2 in 154 nasopharyngeal and throat swab samples collected at Siriraj Hospital with 100% specificity and 100% sensitivity using a fluorescent CRISPR assay, and 100% specific and 97% sensitive with a lateral flow readout.[22] This group demonstrated the CRISPR detection in the LFA format, which is amenable for POC detection. Yet, they followed the cumbersome nucleic acid extraction step, which can be further simplied in the future.[22] Recently, simplified RNA extraction steps for SARS-CoV-2 detection were also demonstrated.

For instance, Joung *et al.* developed a simple assay, SHERLOCK Testing in One Pot (STOP), by incorporating a rapid extraction method of SARS-CoV-2 viral RNA with isothermal preamplification and CRISPR-Cas13a-mediated detection. To extract the viral RNA from the nasopharyngeal or anterior nasal swab, they used an extraction solution containing lysis buffer and magnetic beads, and STOPCovid.v2 reaction mixture was added for target detection. They tested nasopharyngeal swab samples obtained from 402 patients. When compared with the RT-qPCR results, STOPCovid.v2 showed a sensitivity of 93.1% and a specificity of 98.5%. With a simplified nucleic acid extraction procedure, this study provided a promising example for detecting COVID-19 in a POC setting.[65]

In addition, Arizti-Sanz *et al.* developed Cas13-based SHINE[67] and SHINEv2[77] platforms for detecting SARS-CoV-2 from swab samples with 100% specificity. Among these new CRISPR detection platforms with high POC potentials, some excluded the preamplification step (e.g., FIND-IT), while others simplified the nucleic acid extraction procedure (e.g., STOPCovid, SHINE, SHINEv2). As for future perspectives, it would be more ideal if every step of the CRISPR-Dx, from nucleic acid extraction, preamplification, to main reaction itself can be miniaturized in a single platform to make it a truly POC detection method.

While SARS-CoV-2 is an RNA virus, extensive studies have been reported to detect this highly contagious virus using the CRISPR-Cas12a-based DETECTR diagnostic platform. For example, Broughton *et al.* developed a DETECTR-based platform for detecting SARS-CoV-2 from clinical samples. They first extracted nucleic acid from the nasal swabs of 36 patients with COVID-19 infection and 42 patients with other viral respiratory infections. Then, SARS-CoV-2 detection was achieved with 95% positive predictive agreement and 100% negative predictive agreement. This sensing platform provided a visual and faster alternative readout to real-time RT-PCR assay (Fig. 4A).[57]

Ding *et al.* proposed an All-In-One Dual CRISPR-Cas12a (AIOD-CRISPR) assay for low-cost, one-pot, ultrasensitive, and visual SARS-CoV-2 detection from clinical swab samples and obtained consistent results with RT-PCR assay. The AIOD-CRISPR assay helped detect SARS-CoV-2 from clinical swab samples within 20 min. The researchers deidentified 28 clinical swab samples (including 8 COVID-19-positive samples confirmed by real-time RT-AIOD-CRISPR). This platform was able to detect all eight COVID-19-positive samples in a short time.[24] Due to convenient visual readout, CRISPR-Cas12a-based sensing platforms have showed great potential for POC applications.

Pathogen Detection from Plant/Food Samples

CRISPR-Dx strategies have also been used for detecting pathogens from plant samples. Li *et al.* developed a plasmonic CRISPR-Cas12a assay platform utilizing the collateral cleavage activity of Cas12a and plasmon-coupling property of AuNPs upon aggregation. They detected GRBV in red-fruited and white-fruited grapevine samples by the naked eye (Fig. 4C).[6]

Taking similar advantages of CRISPR-Cas12a and AuNPs, Jiao *et al.* reported a strategy of detecting RNA

viruses by using multiplex RT-RPA and distinct LbCas12a/AuNP visual assays. Using different sets of primers for different targets, they first performed RT-RPA in a single tube, and then the amplified targets were detected using different crRNAs in different tubes. This assay platform enabled the detection of ASPV and ASGV with a sensitivity of less than 2.5×10^2 viral copies per reaction. In the case of apple scar skin viroid and apple chlorotic leaf spot virus, the detection sensitivity was 2.5×10^3 viral copies per reaction.[7]

These two colorimetric-based studies showed the huge potential of utilizing the CRISPR technology for detecting pathogens from plant samples in the field settings.

CRISPR technology has also been used to detect pathogens from food samples. For example, WSSV, which causes white spot disease, one of the most severe diseases of cultivated shrimp, can be detected with CRISPR-Dx. Chaijarasphong *et al.* developed a CRISPR-Cas12a system coupled with PCR as a preamplification step for detecting WSSV.[54] For field-level testing, they stored assay components in a freeze-dried paper format and used an inexpensive portable spectrophotometer for signal readout.

Limitation and Future Perspective of CRISPR-Dx

CRISPR-Dx is capable of detecting infectious pathogens with few copies per reaction and distinguishing different subtypes with single base-pair mutations without sophisticated instruments.[21,52] However, there are still a few limitations to be overcome in future studies.

A widely studied CRISPR-Cas12-based detection platform requires the target dsDNA with PAM sequences. As such, fewer options are available when detecting dsDNA pathogens that do not contain PAM sequences. However, this problem was partially solved by altering the PAM specificities by using an engineered Cas12a. For instance, three AsCas12a variants carrying the mutations S542R/K607R, S542R/K548V/N552R, and E174R/S542R/K548R can recognize TYCV, TATV, and VTTV/TRTV/TTYN PAM, respectively (where Y is C or T; and V is A, C, or G; and R is A or G).[91,92] In addition, using PAM-containing primers during the preamplification step in a 1-HOur Low-cost Multipurpose highly Efficient System (HOLMES)-based detection is another way to solve the PAM-related requirement.[93]

Likewise, CRISPR-Cas13-based RNA detection is also limited by the presence of PFS in the target. Although this issue can be solved by selecting the routinely used LwaCas13a enzyme (which does not require PFS),[21] this requirement does constrict the application of CRISPR-Dx to certain targets.

Currently, most CRISPR-Dx require a preamplification step such as RPA, RT–RPA, LAMP, or RT-LAMP to achieve the desired detection sensitivity (e.g., attomolar or femtomolar concentration of targets). However, such a preamplification step complicates the test process, increases the result turnaround time, and is especially challenging for POC implementation. Moreover, the preamplification steps may conceal the true concentrations of analyte in the original samples and therefore influence the accuracy of quantitative results. To address this challenge, several strategies have been developed for sensitive CRISPR-based detection without preamplification. For example, Nguyen *et al.* developed a femtomolar-level detection platform with engineered crRNAs.[72] Shi *et al.* proposed CONAN to detect nucleic acids at greater sensitivity.[73] However, both engineered crRNA and scgRNA need to be specially designed and optimized (e.g., thermodynamically and thermally stable for the CONAN platform).

Fozouni *et al.* constructed an amplification-free CRISPR-Cas13a assay to quantitatively detect RNA of SARS-CoV-2 extracted from clinical samples by combining crRNAs that targeted multiple sites of the viral RNA.[66] However, the main challenge here is to identify multiple highly conserved markers to be recognized by CRISPR in the pathogen genome. Although the machine learning-based ADAPT approach can help solve this issue to some extent for LwaCas13 protein,[74] more research is needed to make the approach universally applicable for many other Cas effectors. The recently demonstrated digiCRISPR assay based on single-molecule counting, on the contrary, could be an alternative method that eliminates the need for preamplification while maintaining sufficient detection sensitivity.[26] However, all of the amplification-free approaches to date are not compatible with unextracted samples.

Sample pretreatment is also one of the major limitations of applying CRISPR-Dx for complex samples such as food or environmental samples. Although the HUDSON protocol solved the problems partially by heating the raw samples (e.g., serum, urine) before target amplification, the overall detection sensitivity was compromised due to the presence of various background molecules in the sample matrix.[75,94] On the contrary, plant sample pretreatment steps can be simplified and fastened by extracting the nucleic acid (both DNA and RNA) directly from infected plant tissues (e.g., leaves) using a polymeric microneedle patch.[80,95,96] The combination of microneedle extraction and CRISPR-Dx could be a promising solution for the rapid detection of plant pathogens in the field. In addition, new CRISPR-Cas effectors with the antifouling feature, robust in raw sample matrix, and providing excellent sensitivity are to be discovered.

Moreover, multiplexed detection is still challenging for CRISPR-Dx. Gootenberg *et al.* utilized the different

cleavage preferences of Cas proteins on reporter nucleotide sequences to develop four multiplex detection mechanisms by using different Cas effectors.[71] However, such methodology is restricted by the number of available Cas enzymes. Therefore, discovering more Cas effectors and understanding sequence preference for *trans*-cleavage of Cas proteins are needed.

Ackerman *et al.* developed the CRISPR-Cas13a-based CARMEN platform for the multiplexed detection of different pathogens. They detected 169 human-associated viruses by creating nanoliter droplets containing amplified samples in microfluidic chips and then tested them against different crRNAs.[64] In this study, the benefit of multiplexing was achieved by compartmentalizing the original samples into different microreactions.

Jiao *et al.* constructed a CRISPR-Cas12a-based RNA plant virus detection platform using a multiplex RT-RPA. This research group separated the amplified samples into five tubes and detected five different disease markers.[7] Still, more work needs to be explored to detect different targets from a single sample. So far, only a few POC CRISPR assays have been demonstrated for multiplexed detection.[97] As for future perspectives, this area may expect more developments in CRISPR-coupled microfluidic chips or LFA devices to convert the multiplexed laboratory CRISPR-Dx into a field-deployable format.

Finally, translating CRISPR reaction into POCTs is emerging but requires further optimization. Several techniques such as isothermal amplification,[21] HUDSON,[75] and visual readouts[7,59] have brought POC features to the CRISPR-Dx. Although a few studies have been reported to demonstrate the CRISPR-Dx in the POC settings,[64] most of them still require a few operations (e.g., preamplification step) separately off the POC device. As such, more progress is needed in this area, which might incorporate microfluidics, biochip, lateral flow, nanotechnology, and smartphone sensor to make CRISPR-Dx more sensitive and better suited to the POC setup. Machine learning and artificial intelligence could be incorporated to design the CRISPR-Dx assay (e.g., guide RNA) swiftly and efficiently. In addition, computational tools can help predict undiscovered Cas effectors with better trans-cleavage efficiency and help understand the fundamental mechanism of trans-cleavage. These findings will unlock the full potential of CRISPR technology for POC and field applications.

Besides, social and psychological factors are also essential to implement a new technology overriding an existing gold standard/widely applied technique (e.g., PCR, antigen–antibody testing). It would be difficult to ask professional users to replace the standard-of-care procedure that has already been used on a large scale and over a long history.[98] Personal and individual users on the contrary may be more willing to try new technologies since nothing similar exists. A desire for rapid home COVID tests is one such example.

Nevertheless, for large-scale deployment, the CRISPR-Dx should be simple enough in operation so that it can be introduced to an individual household or a traditional PCR-based diagnostic laboratory with minimal change of instrumental setup. Along with the immaturity of POC CRISPR-Dx, the adoption of rapid antigen tests[99–101] and these social factors might be why, 2 years into a global pandemic, CRISPR-Dx has not been widely deployed. The parallel efforts in technology breakthroughs and improvement of public awareness/acceptance could soon overcome the technical and social barriers to build another gold standard in diagnostic fields.

Conclusion

Infectious pathogens are pressing global concerns due to the potential pandemic or endemic caused by pathogen infections, which may heavily impact the global health and socioeconomic infrastructure. Rapid, sensitive, specific, and field-deployable detection that can control disease spreading is needed more than ever. CRISPR-Dx approaches (e.g., SHERLOCK, DETECTR, HOLMES, CONAN, CARMEN) and their fast evolution in the past few years have opened a revolutionary horizon in the field of disease diagnostics. CRISPR-Dx has evolved into various signaling mechanisms (e.g., colorimetric, fluorescence-based, electrochemical) to ease the readout. Pathogens from various real-life samples such as human/animal samples, plant samples, and food samples have been successfully tested and detected by CRISPR-Dx. In addition, CRISPR-based detection has been tailored and optimized to make the diagnostic process simpler for POC applications.

However, there are a few remaining issues of CRISPR-Dx, such as the target sequence requirement, sample preamplification and pretreatment, multiplexing, and miniaturization. Despite the remaining challenges, integration of CRISPR with emerging technologies such as nanomaterials, microfluidics, biochip, smartphone device, and machine learning can make the CRISPR-based pathogen detection platforms ideally and practically suitable for field applications. Indeed, the rapid ongoing evolution and development of CRISPR-based biosensors have shown tremendous potential to be used as a next-generation diagnostic method soon.

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