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CRISPR-CAS-BASED TECHNIQUES FOR PATHOGEN DETECTION: ADVANCES, APPLICATIONS, AND FUTURE PERSPECTIVES – A REVIEW

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ABSTRACT

The role of infectious pathogens bacteria in terms of severe human illnesses or even great deaths is undeniable. Over the last few years, the modern lifestyle has raised the problem of infectious pathogen bacteria more and more. Indeed, the rapid, sensitive and selective sensing platforms can prevent the spread of these microorganisms, additionally, optimize medical healthcare systems. In favor of this matter, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system, as a microbial defense system, has attracted considerable attention. To elaborate, although the system protects bacteria from being attacked by invading types, there are various types of developed biosensors using the CRISPR/Cas system for the determination of numerous targets, including bacteria. 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.

INTRODUCTION

Pathogens are organisms that cause diseases in hosts. These organisms are taxonomically diverse and comprise viruses, bacteria, unicellular, and multicellular eukaryotes. Some pathogens can cause large-scale infectious diseases that lead to human morbidity and mortality. Pathogens must be identified quickly to diagnose, cure, and control the associated diseases. Nucleic acid amplification detection tests (NAATs) and next generation sequencing (NGS)-based pathogen diagnostics are the most widely used methods for detecting pathogen . These methods have become the gold standard since they can recognize and differentiate between even a few nucleic acid copies. Furthermore, bacteria categorize into two main groups including Gram-positive (G^+) and Gram-negative (G^-), both of which have a thick cell wall. On the one hand, the thick cell wall of G^+ acts as the outer shell of the cell, on the other hand, G^- bacteria enjoy an outer membrane with some pores and appendages .A

variety of bacteria such as *Escherichia coli (E. coli), Listeria monocytogenes(L.monocytogenes), Salmonellatyphimurium(S.typhimurium),* and *Staphylococcus aureus (S. aureus)* consist of various infectious biological agents which cause severe human illnesses and enormous deaths all over the world Specifically, the modern lifestyle and socioeconomic activities change cause an increased spread rate of infection, resulting escalate the negative consequences of these microorganisms including serious menaces to human healthcare systems and burden abundant economic loss 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 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.

The CRISPR/Cas system is an adaptive immune defense system first discovered in Escherichia coli and used by bacteria and archaea as a defense against virus invasion. CRISPRs refers to clustered regularly interspaced short palindromic repeats, which was first described by Jansen et al. in a study of bacterial and archaeal genome sequences. Its structure is shown in **Figure 1**.

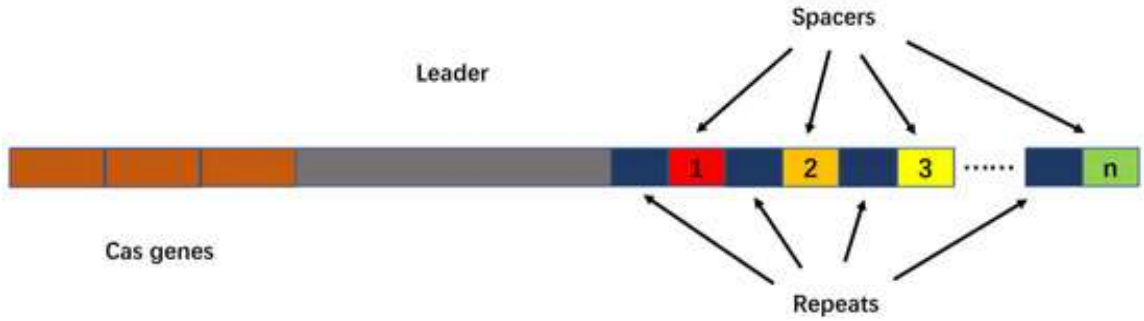


Figure 1. CRISPR-Cas locus structure diagram. CRISPR-Cas includes trans activating crRNA, genes encoding Cas-related proteins (Cas genes), repeat sequences and spacer sequences

CRISPR sequences consist of repeat sequences and spacer sequences. Repeat sequences are repeating palindromic sequences arranged one after the other, separated by spacer sequences, while the DNA of the spacer sequences is not identical.

1.DETECTION TECHNIQUES BASED ON THE WORKING PRINCIPLES OF CRISPR-CAS PROTEINS

Detection methods based on combination with targets

Cas9 can be programmed to target double-stranded DNA (dsDNA) and introduce a double-strand break. The specificity of this detection mechanism is guided by a 20-nucleotide single-guide RNA (sgRNA) that is complementary to the target dsDNA. The application range of Cas9-based detection can be expanded by altering the sgRNA sequence. For successful recognition and cleavage, the Cas protein–sgRNA complex must identify a specific protospacer-adjacent motif (PAM) within the dsDNA, which triggers enzymatic cleavage by the Cas9 protein.

Several CRISPR-Cas9-based methods have been developed for the rapid detection of pathogens such as Zika virus (ZIKV) and *Escherichia coli* (E. coli). A catalytically inactive version of Cas9, termed dCas9, lacks cleavage activity but retains the ability to specifically recognize and bind dsDNA targets. This property has

been exploited in biosensing applications. For example, split luciferase can regain enzymatic activity when its N- and C-terminal halves (NFluc and CFluc, respectively) are reconstituted. Zhang and colleagues fused NFluc and CFluc to the N-terminus of dCas9, enabling luciferase reactivation upon binding of two adjacent target sites in the DNA. In the presence of D-luciferin, this generates a bioluminescent signal, which has been successfully applied to detect *Mycobacterium tuberculosis* (Mtb).

The specificity of this strategy is enhanced by the requirement for two sgRNAs targeting sequences spaced approximately 20 base pairs apart. However, the absence of additional signal amplification limits its sensitivity, necessitating further optimization. Beyond Mtb, dCas9-based approaches have also been adapted for detecting methicillin-resistant *Staphylococcus aureus* (MRSA) and SARS-CoV-2.

Detection methods based on "collateral cleavage" activity

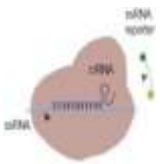
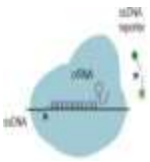
Unlike nucleic acid assays based on CRISPR-Cas9, assays employing CRISPR-Cas12, Cas13, or Cas14 rely on "collateral cleavage" activity that is triggered upon target binding. Cas12 and Cas14 are both RNA-guided DNases; however, Cas12 shows higher specificity for double-stranded DNA (dsDNA), whereas Cas14 exhibits higher specificity for single-stranded DNA (ssDNA). These nucleases not only recognize and cleave dsDNA or ssDNA sequences with the guidance of CRISPR RNA (crRNA), but also indiscriminately degrade surrounding non-target ssDNA reporters.

Cas12-based diagnostic platforms such as the one-Hour Low-cost Multipurpose highly Efficient System (HOLMES) and the DNA Endonuclease Targeted CRISPR Trans Reporter (DETECTR) have demonstrated reliable detection of pathogens, including human papillomavirus (HPV) and Japanese encephalitis virus. DETECTR was further adapted for the rapid diagnosis of SARS-CoV-2 by integrating loop-mediated amplification (RT-LAMP) with Cas12-mediated collateral cleavage, creating the SARS-CoV-2 DETECTR platform. Another notable innovation, the POC-CRISPR system, combined Cas12 with reverse transcription and recombinase polymerase amplification (RT-RPA), employing droplet magneto fluidics to enable point-of-care SARS-CoV-2 detection. This portable device integrates simplified bead-based sample preparation, nucleic acid amplification, Cas12 reaction, and real-time result reporting, achieving detection directly from nasopharyngeal swab samples in under 30 minutes.

Cas14 represents a unique class of CRISPR effectors, discovered primarily in members of the extreme biophilic DPANN superphylum. Unlike most Cas proteins, which range from 950 to 1400 amino acids in length, Cas14 is remarkably small, spanning only 400–700 amino acids, making it the smallest RNA-directed nuclease identified to date. Cas14 can specifically recognize and cleave ssDNA through a seed sequence interaction located near the center of the target sequence. In vitro studies using purified Cas14a-sgRNA complexes have confirmed their ability to mediate RNA-guided ssDNA cleavage with high specificity, including discrimination of single nucleotide polymorphisms (SNPs).

While Cas9, Cas12, and Cas14 are primarily suited for the detection of DNA targets, Cas13 functions as an RNA-guided ribonuclease, capable of detecting RNA-type nucleic acids. Cas13 generates multiple cleavage sites within single-stranded regions of RNA targets and, notably, remains catalytically active even after cleavage. Similar to Cas12 and Cas14, Cas13 also exhibits collateral cleavage activity, enabling the degradation of nearby bystander RNAs in a target RNA-dependent manner.

Table 1. Overview of detection properties of different CRISPR proteins.

Effector protein	**Cas9**	**Cas12**	**Cas13**	**Cas14**
PAM required?	Yes	Yes	No	No
Target type	dsDNA	(ds/ss)DNA	ssRNA	(ss/ds)DNA
Collateral cleavage	No	ssDNA	ssRNA	ssDNA
Action diagram				
Application	NASBACC	DETECTR, HOLMES	SHERLOCK	DETECTR-Cas14

2.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.

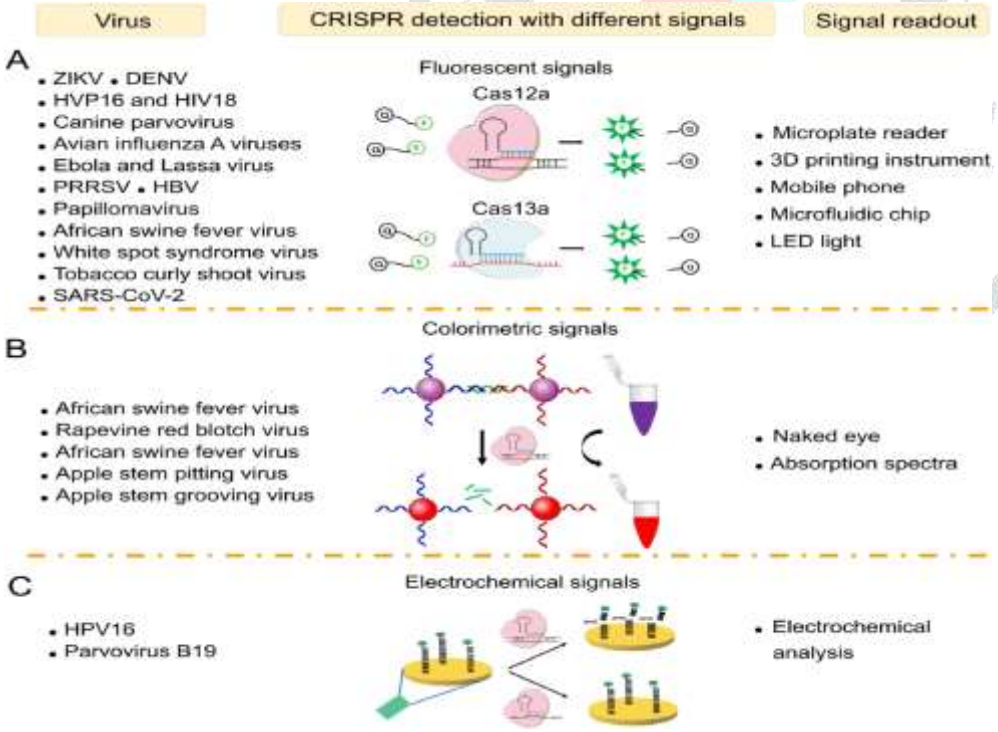
Virus sensing with different signal readouts

2.1. Virus sensing via fluorescent signals

The first two CRISPR-based sensing platforms, SHERLOCK and DETECTR, were originally developed using fluorescence readouts. In these systems, short single-stranded nucleic acid reporters were doubly labeled with a fluorophore at the 5′ end and a quencher at the 3′ end. Upon recognition and binding to the target RNA or DNA, the collateral cleavage activity of Cas13a or Cas12a was activated, leading to nonspecific cleavage of the reporter. This process separated the fluorophore from the quencher, thereby generating a detectable fluorescence signal.

SHERLOCK demonstrated the ability to detect viruses such as Zika virus and dengue virus with extremely high sensitivity at the attomolar level and even single-base resolution. To adapt the assay for field deployment, a paper-based version known as SHERLOCKv2 was developed, which incorporated freeze-dried Cas13a. Although the sensitivity was slightly reduced compared to the original platform, detection limits as low as 20 attomolar were still achieved. DETECTR, on the other hand, was successfully applied for the detection and differentiation of human papillomavirus subtypes by targeting the hypervariable regions of the L1 gene using specific crRNAs.

Beyond these initial applications, CRISPR-Cas12a and Cas13a platforms have been utilized for the detection of a wide range of pathogens, including canine parvovirus, avian influenza virus, porcine reproductive and respiratory syndrome virus, hepatitis B virus, African swine fever virus, white spot syndrome virus, Japanese encephalitis virus, tobacco curly shoot virus, and several haemorrhagic



viruses such as Ebola and Lassa. Importantly, these approaches were rapidly adapted for SARS-CoV-2 detection, where near single-copy sensitivity was reported with fluorescence readouts. Furthermore, CRISPR-based assays were capable of identifying drug-resistance mutations in viruses such as HIV and HBV by employing mutation-specific crRNAs.

The sensitivity and specificity of Cas12a- and Cas13a-mediated detection can be further enhanced by incorporating auxiliary enzymes such as Csm6, by designing engineered crRNAs, or by employing multiple crRNAs targeting different regions of the pathogen genome. Fluorescence-based detection systems generally offer advantages such as simplicity, low background noise, and a high signal-to-noise ratio. In addition to

conventional fluorescence plate readers, alternative platforms have been integrated for point-of-care testing, including smartphone-based detectors, LED blue-light devices, portable instruments, and microfluidic chips. These adaptations have significantly broadened the applicability of CRISPR fluorescence assays for field diagnostics and rapid screening.

3.CRISPR-BASED BIOSENSOR IN DIAGNOSIS (DISEASES)

Emerging Infectious Pathogens and CRISPR-Based Diagnostics

The rise of infectious pathogens has become a major global health threat. In recent years, outbreaks such as Zika virus, Ebola, Influenza A (pH1N1), Middle East respiratory syndrome coronavirus, and most recently the COVID-19 pandemic, have shown how rapidly diseases can spread in a densely populated world [53,54]. Although conventional diagnostic approaches, including culture methods and biochemical analyses, have contributed significantly to disease detection, they are often time-consuming, labor-intensive, and not always effective for rapid clinical response [55]. The emergence of drug-resistant pathogens has further complicated this scenario, highlighting the urgent need for faster and more reliable diagnostic systems [56].

CRISPR-Cas systems offer a unique solution to this challenge. These programmable nucleases can precisely target and cleave DNA or RNA sequences, guided by crRNA molecules that recognize pathogen-specific nucleic acids [57]. Leveraging this property, diagnostic tools such as DETECTR and SHERLOCK were developed, alongside newer platforms like NASBACC, SHERLOCKv2, and HOLMES [58,59]. These technologies combine sensitivity, speed, and adaptability, making them well-suited for pathogen detection and point-of-care applications.

3.1. Bacterial Pathogens

3.1.1. Foodborne Pathogens (Escherichia coli, Salmonella, Staphylococcus aureus)

Food safety is a critical global concern, as consumption of contaminated or undercooked food can expose individuals to pathogens causing serious illnesses. CRISPR-based biosensing has emerged as a promising tool for detecting bacterial nucleic acids in food samples with greater sensitivity and speed [60,61].

One example is a CRISPR-Cas12a (Cpf1) system integrated with a hybridization chain reaction (HCR) for detecting **Salmonella typhimurium**, a common cause of foodborne infections [62,63]. Another platform, Cas12a-Ddp, allows dual detection of pathogenic and drug-resistance genes with high sensitivity, achieving limits of detection as low as 1 CFU/mL in under 40 minutes [64].

For **E. coli O157:H7**, CRISPR-Cas9 has been applied in combination with rolling circle amplification and strand displacement methods, resulting in highly sensitive fluorescence-based detection [65,66]. Similarly, a CRISPR-Cas13a method known as **CCB-Detection** was developed for **Staphylococcus aureus**, showing high specificity and faster results compared to conventional culture-based methods [66,67].

3.1.2. Tuberculosis

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains one of the leading causes of death worldwide, second only to COVID-19 in terms of infectious disease burden [68]. Advances in biosensor technologies, including CRISPR-based diagnostics, have significantly improved the ability to detect TB with high sensitivity and shorter turnaround times.

The **CRISPR-MTB test** utilizes the Type III-A CRISPR system of *M. tuberculosis* and has demonstrated near single-copy sensitivity. When compared with conventional culture and GeneXpert methods, CRISPR-MTB showed enhanced performance, making it a strong candidate for both pulmonary and extrapulmonary TB diagnostics [69–71].

3.1.3. Chlamydia

Chlamydia trachomatis is among the most common sexually transmitted infections worldwide. The presence of CRISPR systems in *Chlamydia* was first described with subtype I-E loci containing Cas-Cse genes [72].

Using CRISPR interference (CRISPRi) with dCas9, researchers were able to suppress expression of specific genes such as **incA** without affecting bacterial growth [73]. More recently, dCas12 systems have been applied, expanding the range of targetable genes and enabling functional studies in *Chlamydia* [74].

3.1.4. Helicobacter pylori

Helicobacter pylori is a Gram-negative bacterium strongly associated with gastric ulcers and gastric cancer. Traditional diagnostic methods—ranging from invasive endoscopy to non-invasive urea breath and serological tests—have limitations in terms of accuracy, reliability, and cost [75–86].

Biosensors have been increasingly applied for *H. pylori* detection, including fluorescent DNA sensors, electrochemical DNA-based platforms, and paper-based colorimetric devices [87–93]. Recently, CRISPR-Cas12a has been adapted into a lateral flow biosensor for stool sample testing. This method achieved a detection limit of just 5 copies/μL and was able to distinguish *H. pylori* from other gut pathogens with high specificity [96,97]. The simplicity and portability of such CRISPR-based assays make them highly promising for point-of-care applications.

3.2. Viral Pathogens

3.2.1. Hepatitis Viruses

Hepatitis viruses (A–E, G) remain a significant global burden, with hepatitis B and C being the major causes of liver cancer and cirrhosis. An estimated 354 million people worldwide live with chronic HBV or HCV infection [98–100]. Current diagnostic techniques, including PCR and serological assays, are effective but often slow, expensive, and not always suitable for large-scale screening [101,102].

CRISPR-based diagnostics are beginning to transform this field. For example, the **CRISPR-HBV test**, based on Cas12b and a gold nanoparticle lateral flow biosensor, enables rapid, highly specific detection of HBV genotypes within an hour [108]. Colorimetric CRISPR-Cas12a assays, PCR-Cas13a combinations, and amplification-free SERS-based sensors have also been developed, all showing high sensitivity for HBV detection [109–111]. Such platforms provide new opportunities for cost-effective, rapid point-of-care testing.

3.2.2. Human Immunodeficiency Virus (HIV)

HIV remains a major global health issue, with nearly 38 million people living with the infection [112]. CRISPR tools, including Cas9, Cas12a, and Cas13a, have been explored both for potential therapeutic strategies and as diagnostic platforms.

Cas9 and Cas12a systems have been applied to excise or deactivate proviral DNA, while Cas13a has been shown to inhibit viral RNA replication. These approaches not only improve diagnostic potential but also offer novel antiviral strategies with promising implications for future HIV management [113,114].

3.2.3. Dengue Virus

Dengue virus (DENV), transmitted by *Aedes aegypti* mosquitoes, is a major public health concern in tropical and subtropical regions [115]. CRISPR-Cas9 and Cas13a platforms have been applied for genetic screening and inhibition of dengue replication. By targeting host and viral factors, these systems enable highly specific and efficient genome editing, paving the way for novel diagnostics and control strategies [116,117].

3.2.4. Human Papillomavirus (HPV)

HPV is the primary cause of cervical cancer, responsible for over 300,000 deaths annually, particularly in low- and middle-income countries [118]. Conventional cytology and PCR-based screening methods have limitations in sensitivity, accessibility, and cost.

CRISPR-Cas12a systems integrated with RPA amplification and lateral flow readouts have been developed for HPV detection. These methods demonstrated high sensitivity (as low as 0.24 fM) and the ability to distinguish between HPV16 and HPV18 strains in clinical samples [119–121]. Compared to traditional assays, CRISPR-based platforms offer faster, cheaper, and more portable solutions for HPV screening.

Table 1. Overview of detection of pathogens along with the Cas proteins.

Pathogen	CRISPR	Nucleic Acid	Amplification	Readout	LOD	Detection Platform	Ref.
Food poisoning (*E. coli*, *Salmonella*, *S. aureus*)	Cas9a	DNA	SDA	Colorimetric	100 copies	-	[122]
	Cas12a	dsDNA	HCR	Electrochemical	20 CFU/mL		[122]
	Cas12a-Ddp	dsDNA	PCR	Microplate	1 CFU/mL	Dual detection platform	[122]
	Cas13a	ssRNA	PCR	Florescence	1 CFU/mL	CCB-Detection	[122]
Tuberculosis	dCas9	DNA	PCR	Bioluminescence	≈3 × 10^{-21} M	Chimeric dCas9-luciferase	[123]
	Cas12a	DNA	RPA	Florescence	1 copy	CRISPR-MTB	[123]
H. pylori	Cas12a	DNA/RNA	RPA	Lateral flow strips, visualization	5 copies/μL	Lateral flow biosensor	[124]
Hepatitis Liver cancer	Cas12a	DNA/RNA	HCR	Gel electrophoresis	1.5 fM	-	[125]
	Cas12a	DNA/RNA	LAMP	Colorimetric	10 aM	AuNP colorimetric	[126]
	Cas12a	DNA	-	Colorimetric	10 pM	MAV-chip	[127]

4. CRISPR-CAS9 TECHNOLOGY FOR PATHOGEN DETECTION

In the CRISPR-Cas9 system, invasion by exogenous bacterial DNA initiates the transcription of the CRISPR sequence to produce tracrRNA and pre-crRNA. The pre-crRNA is processed into mature crRNA by ribonuclease III. This crRNA binds with tracrRNA to form a single-guide RNA (sgRNA). The sgRNA directs the Cas9 protein to the protospacer adjacent motif (PAM) sequence located at the 3′ end of the exogenous DNA and unwinds the double-stranded DNA. Cas9, which contains the HNH and RuvC-like nuclease domains, cleaves both complementary and non-complementary DNA strands, resulting in a double-strand break (DSB) at the target locus. The DSB activates two repair mechanisms: non-homologous end joining (NHEJ) and homology-directed repair (HDR), both of which facilitate genome editing .

CRISPR-Cas9 was first applied in nucleic acid detection in 2016. Collins et al. combined nucleic acid sequence-based amplification (NASBA) with the CRISPR-Cas9 system to develop a detection platform capable of identifying different subtypes of the Zika virus (ZIKV). NASBA was used to reverse transcribe and amplify RNA into dsDNA, which was subsequently detected by Cas9-sgRNA complexes targeting specific viral subtypes. The detection results could be visualized directly through colour change observable by the naked eye.

Subsequent developments expanded the application of CRISPR-Cas9 in diagnostics. Wang et al. established the CRISPR-Cas9 mediated lateral flow nucleic acid assay (CASLFA), which successfully detected *Listeria monocytogenes* within one hour. Sun et al. designed a fluorescent detection method for *Escherichia coli* O157:H7 using a Cas9-sgRNA binary complex that triggered strand displacement amplification and rolling circle amplification, providing a highly sensitive readout.

Further advancements have been made by modifying Cas9. When mutations were introduced in both the HNH and RuvC-like nuclease domains, a deactivated form of Cas9 (dCas9) was obtained. Although dCas9 lost its DNA cleavage ability, it retained high binding specificity, making it suitable for pathogen detection. Zhang et al. utilized this property to establish an *in vitro* system for detecting *Mycobacterium tuberculosis*. Similarly, Kim et al. integrated CRISPR-Cas9 with surface-enhanced Raman scattering (SERS) for detecting multidrug-

resistant bacteria ,while Guk et al. combined DNA fluorescent in situ hybridization (FISH) with CRISPR-Cas9 to construct a DNA-FISH system based on dCas9/sgRNA-SYBR Green I, enabling successful detection of methicillin-resistant *Staphylococcus aureus* (MRSA) .

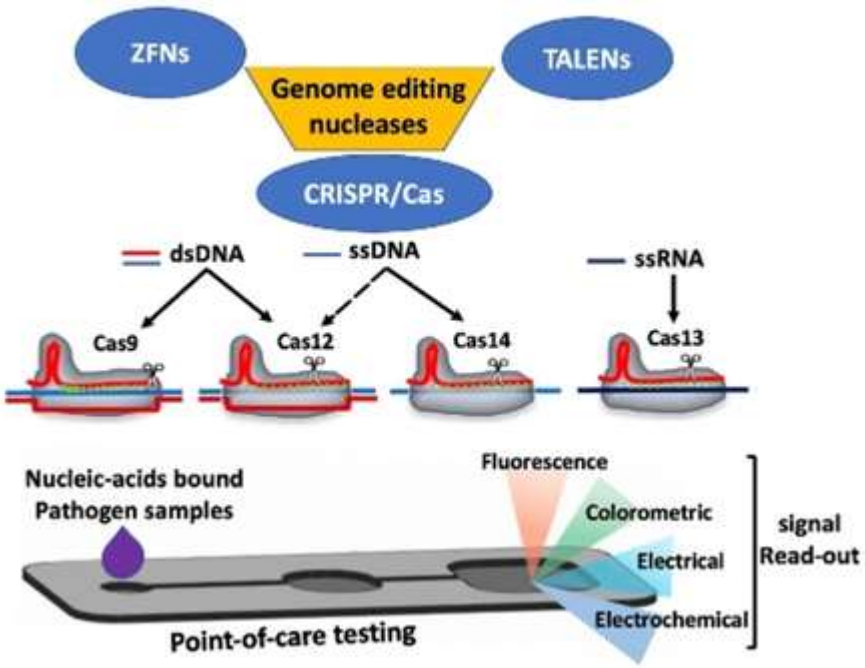


Fig 4,1Point-of-Care Pathogen Detection with CRISPR-based Programmable Nucleic Acid Binding Proteins

5 DETECTION PRINCIPLES AND SIGNAL OUTPUT METHODS OF LATERAL FLOW ASSAY

5.1 PRINCIPLE OF LATERAL FLOW ASSAY

The lateral flow assay (LFA) is a rapid detection method based on *in vitro* immune response principles, widely used for detecting specific biomolecules or target sequences such as proteins, DNA, or RNA. This technique employs paper or membrane materials embedded with specialized antibodies or oligonucleotide sequences. Compared to conventional laboratory instruments, LFA is cost-effective, portable, and user-friendly. It eliminates the need for specialized personnel, complex equipment, or intricate sample preparation, allowing for rapid and straightforward detection.

An LFA strip consists of four primary components: the sample pad, where the sample is applied; the conjugate pad, which contains the biorecognition element and its associated label; the test pad, typically made of nitrocellulose, which includes the test line (T) and control line (C) for target-DNA/probe DNA hybridization or antigen–antibody interactions; and the absorbent pad, which collects excess sample. LFA strips usually employ colorimetric or fluorescent tags as signaling elements, producing readable outputs that can be qualitative or on/off. These results can be observed directly with the naked eye or measured using devices such as spectrometers or smartphones.

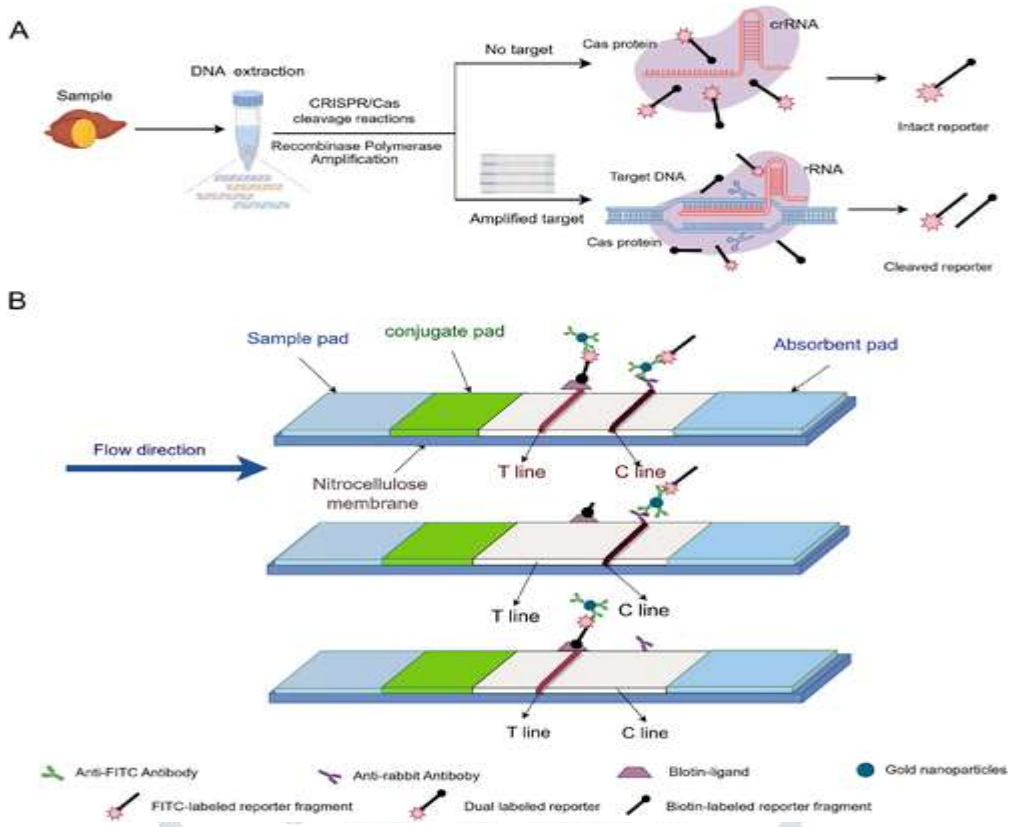


Fig 5.1 Schematic diagram of RPA-Cas-LFB method (By Figdraw).

6. SARS-COV-2 (SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2)

The 2019 pandemic-causing coronavirus disease, also known as COVID-19 (SARS-CoV-2), was initially detected in China on 31 December 2019. SARS-CoV-2 spread rapidly over the world, prompting the World Health Organization (WHO) to proclaim on 30 January 2020, that the COVID-19 outbreak was a public health emergency of international significance. The major routes of transmission are respiratory droplets; SARS-CoV-2 can be transmitted to a healthy person if they comes into contact with an infected person or any of their things, such as clothes, doorknobs, etc. Aerosol transmission (airborne transmission) of this virus has also been observed in research studies Individuals infected with a novel variant ofSARS-CoV-2 have a wide range of symptoms, ranging from asymptomatic to acute respiratory distress syndrome and multi-organ failure. As a result, diagnosing COVID-19 accurately has proven to be difficult.

COVID-19 has so far been diagnosed using associated symptom analysis and a variety of laboratory detection methods, which include nucleic acid amplification tests (NAAT), computed tomography (CT) scans, and serological procedures. Molecular and serological testing are the two types of analytical diagnostic procedures for COVID-19 tests that are currently accessible and **Figure 3** depicts the methodology on detecting COVID-19 using a CRISPR-based detection system. The reverse transcription-polymerase chain reaction (RT-PCR)-based approach is used in the first category of molecular tests for the detection of SARS-CoV-2 virus RNA. Other approaches, such as isothermal nucleic acid amplification tests, transcription-mediated amplification, and CRISPR-based technologies, offer potential alternatives to RT-PCR testing. Here we mostly review alternative PCR technologies that involve the use of CRISPR Cas systems.

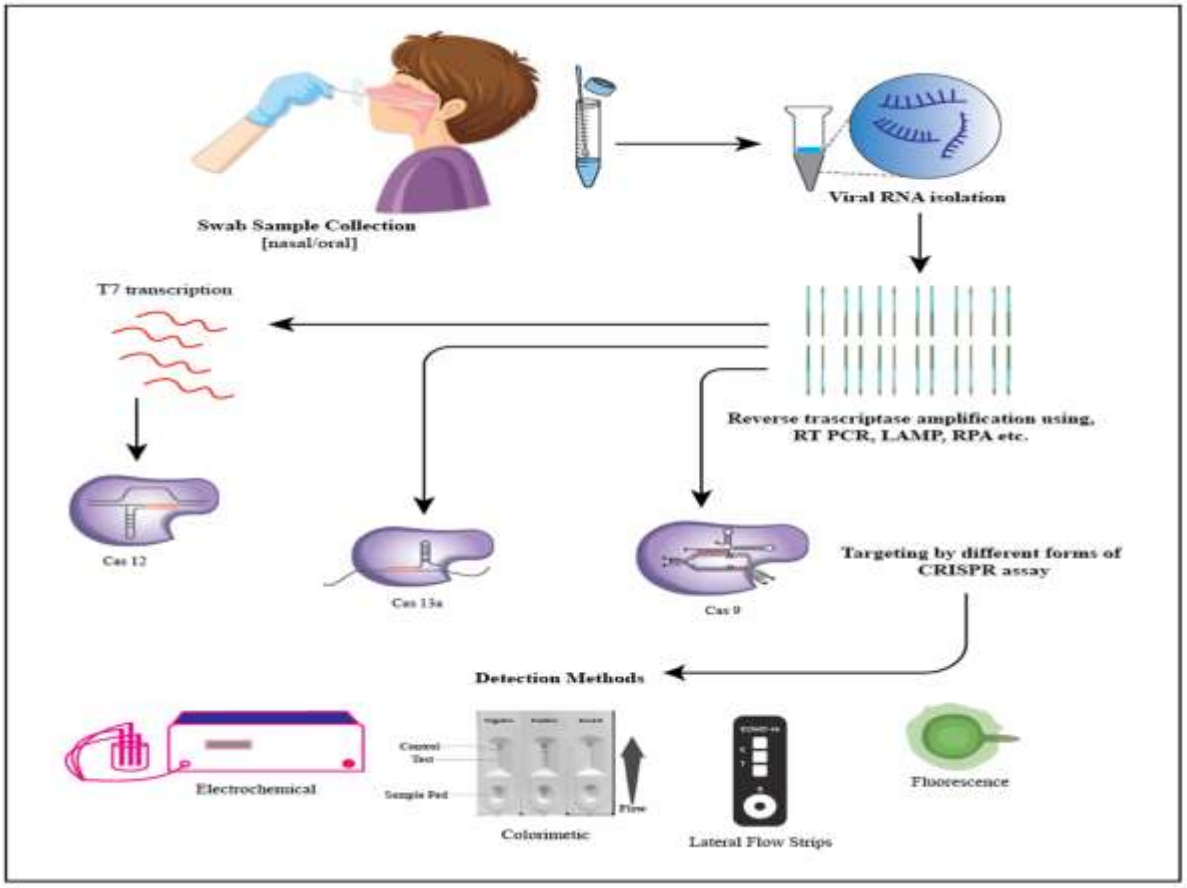


Figure 3. Five-step workflow of CRISPR-based detection system to detect SARS-CoV-2 virus. (1) Sample collection using nasal or oral swabs. (2) RNA molecules are extracted and isolated from the sample, as SARS-CoV-2 is an RNA-based virus. (3) Isolation is followed by an amplification procedure such as RT-PCT, RT-RPA, or RT-LAMP to increase the LOD, (4) followed by detection using the CRISPR-Cas system and (5) detection using fluorescence, colorimetry, electrochemical, or other sensory readout methods

The samples are gathered from the patients in the first stage. A healthcare professional could collect them using Dacron or polyester flocked swabs. For SARS-CoV-2 detection, saliva specimens have recently been added as something of an alternative sample source .

Viral RNAs must be isolated from the raw sample once it has been collected. RNA isolation processes usually consist of three steps: sample cell lysis; RNA isolation from other biomolecules such as DNA, proteins, and lipids; and finally RNA elution [142]. One of these three major devices can be used to separate viral RNA molecules.

1. Spin column-based NAE (nucleic acid extraction) provided the most suitable option for RNA isolation. It plays an important role in ion exchange methods because it offers a stable stationary phase for fast and reliable buffer exchange .

2. Purification with magnetic beads. Magnetic beads are used to collect viral RNA in this procedure. During the wash and collection, the beads are held in place by an external magnetic field. The magnetic format enables quick sample collection and concentration. Manually capturing and releasing particles, on the other hand, might be time-consuming.

3. Extracting organically. In this method, a phenol-containing solution is used to homogenize the specimens, which are then centrifuged. The viral RNA is found in the top aqueous phase during centrifugation. The viral RNA is isolated from the upper aqueous phase and recovered during centrifugation, precipitating it using alcohol (elution) and later placing it in a rehydrating medium. This approach is regarded as the finest for RNA extraction; nevertheless, it is tedious, time-consuming, and difficult to automate.

RNA isolation is usually followed by an amplification procedure such as RT PCT, RT RPA, or RT-LAMP to increase the LOD. Following that, the Cas-CRISPR RNA (crRNA) complex will detect a particular area of SARS-CoV-2. The amplification primers and crRNAs should target the same SARS-CoV-2 locus. Fluorescence- or colorimetric-based devices were the most frequent techniques for signal reading. The CRISPR

assay may be included in lateral flow readout strips as well [144]. So far, the vast majority of tests developed have used the Cas12 or Cas13 family of CRISPR proteins as CRISPR effectors. **Table 6.1** shows the CRISPR protein that is capable to detect COVID-19.

CRISPR Effector Protein	Targeted Gene	Preamplification Method	Signal Readout	Assay Reaction Time	LOD	Reference
Cas9a	N1, N2, and N3 genes	RT-PCR	Colorimetric	Not reported	140 pM	[145]
Cas12a	ORF1ab	RT-RPA	Colorimetric and fluorescence	1 h	10 copies/μL	[146]
Cas12a	N gene	RT-RPA	Fluorescence	40 min	10 copies/μL	[147]
Cas12a	N gene and E gene	RT-LAMP	Colorimetric	30–40 min	10 copies/μL	[148]

Table 6.1. Overview of CRISPR protein that can be used to detect SARS-CoV-2 virus.

7.ADVANTAGES OF CRISPR-BASED PATHOGEN DETECTION

CRISPR-based diagnostics have revolutionized pathogen detection due to their **high specificity and sensitivity**. The system's ability to target nucleic acids precisely via guide RNA allows for discrimination between closely related strains or single-nucleotide polymorphisms. Unlike conventional detection methods such as PCR or culture-based techniques, CRISPR assays can provide **rapid results**, sometimes within minutes to an hour, making them suitable for point-of-care testing (POCT).

Another advantage is the **versatility of CRISPR proteins**. Cas9, Cas12, Cas13, and Cas14 can detect DNA or RNA targets, expanding the detection range across bacterial, viral, and fungal pathogens. Cas12 and Cas13 possess collateral cleavage activity, which enables signal amplification without complex equipment, while lateral flow assays or fluorescence readouts provide **user-friendly, visual results**.

CRISPR-based systems are also **cost-effective and portable**, especially when integrated with paper-based assays or simple fluorescent devices. Minimal sample preparation is required, reducing labor and resource requirements. Furthermore, CRISPR technology can be adapted quickly to emerging pathogens, as the target sequence can be easily modified by designing new guide RNAs.

 High specificity: CRISPR systems use guide RNA (sgRNA or crRNA) to recognize precise nucleic acid sequences, allowing them to distinguish closely related pathogen strains or even single-nucleotide variations.

 High sensitivity: Collateral cleavage activity of Cas12 or Cas13 enables signal amplification, allowing detection of very low levels of nucleic acids, often down to attomolar concentrations.

 Rapid detection: CRISPR-based assays, especially when combined with isothermal amplification techniques like RPA or LAMP, can deliver results in under an hour, much faster than conventional culture-based methods.

 Versatility: CRISPR systems can detect DNA or RNA viruses, bacteria, and other pathogens, making them applicable across a wide range of infectious agents.

 Minimal equipment requirement: Many CRISPR-based tests can be read visually using lateral flow strips or simple fluorescence devices, making them suitable for point-of-care and resource-limited settings.

 Multiplexing potential: Using multiple guide RNAs or Cas proteins, CRISPR assays can detect several pathogens simultaneously in a single test.

 Portability: CRISPR-based diagnostics can be integrated into handheld or portable devices, allowing field deployment and real-time monitoring during outbreaks.

 Cost-effectiveness: Compared to conventional laboratory-based diagnostics, CRISPR-based assays reduce the need for expensive reagents and instrumentation.

 Adaptability: By simply redesigning the guide RNA, the system can be quickly reprogrammed to detect emerging pathogens or new strains.

 Compatibility with novel biosensors: CRISPR can be integrated with electrochemical, optical, and nanomaterial-based biosensors, further improving detection limits and readout options.

 Non-invasive sample analysis: Some CRISPR assays can be applied directly to saliva, urine, or stool samples, reducing discomfort and simplifying sample collection.

8. FUTURE PROSPECTS

Timely detection and accurate assessment of infections are crucial for controlling and eradicating diseases effectively. Current diagnostic technologies, however, face limitations such as high cost, lower sensitivity, and long detection times. CRISPR-Cas sensor technology has emerged as a promising solution to some of these issues, offering reliable results with high specificity. By targeting and cleaving specific DNA or RNA sequences, CRISPR-Cas systems achieve greater accuracy, sensitivity, and specificity compared to traditional detection methods, making them well-suited for point-of-care applications.

Different CRISPR-Cas proteins have distinct diagnostic properties. For example, Cas12b can differentiate individual bases, while Cas14 can identify single nucleotide polymorphisms, such as those responsible for eye color changes, a task that Cas12 cannot perform. Over time, CRISPR technology has evolved from a simple DNA/RNA cleaving tool to a versatile platform for gene editing, biosensing, and diagnostics, demonstrating its growing potential in both fundamental and applied research. CRISPR-based methods also overcome the low sensitivity problem often seen in conventional detection, with some systems capable of detecting attomolar levels of target molecules, far surpassing PCR-based methods.

Another key advantage of CRISPR is its adaptability: changing the guide RNA allows the detection of virtually any nucleic acid sequence, enabling the rapid identification of diverse pathogens. This capability allows clinicians and researchers to quickly evaluate infections and choose targeted treatments. Despite these benefits, most CRISPR-based sensors currently focus on nucleic acid targets. Challenges remain in extending this technology to non-nucleic acid detection due to the reliance on collateral cleavage for signal generation.

Other limitations include oversensitivity, potential off-target activity, and false positives caused by CRISPR's tolerance for mismatched sequences. Most CRISPR-based point-of-care solutions, except for SHERLOCK (FDA-approved in 2020 for SARS-CoV-2), are not yet widely implemented in healthcare, unlike established methods such as RT-PCR. Sample pre-treatment is another hurdle, particularly in food testing or field applications, though methods like HUDSON have helped streamline the process for RNA/DNA targets. For in vivo applications, the complex cellular environment reduces CRISPR efficiency, highlighting the need for tools that guide the CRISPR complex to its target within cells.

Despite these challenges, CRISPR-Cas-based biosensing continues to advance steadily, offering rapid, portable, and reliable detection.

9.CHALLENGES

Nucleic acid detection using CRISPR/Cas biosensors offers several advantages, including high specificity, excellent sensitivity, and simple operation. In some cases, these methods do not require sophisticated instruments. They can detect even trace amounts of viral material and differentiate between subtypes or mutations. Moreover, CRISPR-based assays can be integrated with various technologies to meet the demands of different applications. Despite these benefits, there are several challenges that need to be addressed for practical implementation.

One limitation arises from sequence requirements. Certain CRISPR/Cas effectors, such as Cas12a, rely on protospacer adjacent motif (PAM) sequences to recognize target DNA. While this enhances specificity, it restricts the choice of target sites. Detecting short sequences or single-nucleotide polymorphisms can therefore be challenging. Strategies have been developed to overcome this, such as incorporating PAM sequences into PCR products using specialized primers or designing LAMP primers containing PAM sites, enabling the detection of sequences that would otherwise be inaccessible.

Multiplexing and quantitative detection also present difficulties. Methods like SHERLOCKv2, which use multiple Cas proteins to target different reporters, allow simultaneous detection of several targets but require precise control over protein amounts to avoid cross-reactivity. Other approaches, such as microwell array-based ARMEN or microfluidic combinatorial arrays, have enabled the detection of multiple pathogens, including SARS-CoV-2, in a single assay. Quantitative detection is further complicated by high amplification efficiency, which can saturate reporters and distort measurements of high-concentration targets. Pre-amplification, often necessary for CRISPR detection, can also affect the true concentration of nucleic acids, making accurate quantification difficult.

Sample preparation is another critical aspect. Complex samples often require pre-treatment to extract nucleic acids, but this can introduce errors or reduce sensitivity. Amplification steps may also introduce mutations, and contamination during sample handling can produce false positives. Efforts have been made to detect non-amplified targets directly, although sensitivity tends to be lower compared to amplified detection. Future developments should focus on simpler extraction methods, robust Cas effectors suitable for complex samples, and the ability to detect targets without amplification.

Operational contamination remains a concern. Many CRISPR-based assays involve two steps—pre-amplification followed by detection—exposing the system to environmental RNases or other contaminants. Integrating amplification and detection into a single-step reaction has been demonstrated for SARS-CoV-2 detection, reducing contamination risk and simplifying procedures.

For practical deployment, CRISPR/Cas technologies need to be optimized for real-world applications. This includes improving sample handling, reducing contamination, enabling accurate quantification, and developing more efficient signal detection methods. Additionally, uniform standards are lacking. Reaction conditions, such as component concentrations, temperature, timing, nucleic acid extraction methods, and signal readout, vary between studies and can affect results. Establishing standardized protocols will be essential for point-of-care testing, food safety monitoring, and other practical applications.

10.CONCLUSION

In conclusion, CRISPR-Cas-based biosensors have emerged as powerful tools for pathogen detection, offering unmatched specificity, sensitivity, and flexibility. Their ability to detect even minimal amounts of viral or bacterial nucleic acids, distinguish between subtypes, and identify single-nucleotide changes makes them highly effective for early diagnosis and outbreak control. The adaptability of CRISPR systems allows integration with amplification techniques, lateral flow assays, and microfluidic platforms, creating portable and user-friendly devices suitable for point-of-care applications. Compared to traditional methods, these technologies are faster, more accurate, and often require minimal equipment, making them especially valuable in resource-limited settings.

Despite these advantages, challenges remain that need careful consideration. Dependence on PAM sequences can limit target options, multiplex detection requires careful optimization to prevent interference, and sample pre-treatment or multi-step procedures can introduce contamination or reduce accuracy. Additionally, the majority of CRISPR biosensors focus on nucleic acids, while detection of other biomolecules remains underdeveloped. Standardized protocols for reaction conditions, sample handling, and signal interpretation are also lacking, which can hinder widespread adoption.

Ongoing research is addressing these limitations through innovations such as one-step reactions, robust Cas effectors, and improved signal-readout strategies. With continued refinement, CRISPR-based diagnostics have the potential to transform rapid testing, point-of-care monitoring, and personalized treatment approaches. As these technologies mature, they are poised to become a cornerstone of modern biosensing, enabling faster, more reliable, and cost-effective detection of pathogens across clinical, environmental, and food safety applications.

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