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Review

CRISPR-Cas-mediated diagnostics

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An ideal molecular diagnostic method should be sensitive, specific, low cost, rapid, portable, and easy to operate. Traditional nucleic acid detection methods based mainly on PCR technology have not only high sensitivity and specificity, but also some limitations, such as the need for expensive equipment and skilled technicians, being both time and labor intensive, and difficult to implement in some regions. However, with the continuous development of CRISPR-Cas technology and its application in molecular diagnosis, new approaches have been used for the construction of molecular diagnostic systems. In this review, we discuss recent advances in CRISPR-based molecular diagnostic technologies and highlight the revolution they bring to the field of molecular diagnostics.

Highlights

CRISPR-Cas-mediated diagnostics can detect various pathogens, cancer biomarkers, and SNPs with high specificity, speed, low cost, and portability.

The combination of Cas proteins with biosensors, biochips, biomagnetic beads, isothermal amplification, lateral flow, protein aptamers, and other technologies has resulted in the development of new diagnostic methods.

Multiplexed CRISPR nucleic acid detection systems complete the detection of 169 viruses for diagnosing different diseases in a single sample, with the help of microfluidic technology.

CRISPR-mediated antibody diagnosis technology can recognize thousands of antibodies in a sample simultaneously.

The potential of CRISPR-Cas biosensing technologies is inspiring researchers to develop next-generation diagnostics platforms.

Infectious disease threats and emerging diagnostic strategies

Infectious diseases have long been recognized as an inevitable public health problem. For example, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to significantly impact public health across the world [1–4]. Many traditional PCR-based or qPCR-based nucleic acid detection methods have been used for clinical testing, but the diagnosis process is costly, is time and labor intensive, and requires skilled personnel [5,6]. Thus, such methods are unsuitable for the rapid detection of some infectious diseases [7]. Therefore, rapid, sensitive, low-cost, portable, and easily operated detection systems are essential for the control and management of infectious diseases [8,9]. CRISPR-Cas diagnosis technology has attracted attention because of its advantages of high speed, high efficiency, portable equipment that is easy to use, and low cost [10].

CRISPR-Cas systems comprising **guide RNA** (**gRNA**; see Glossary) and Cas protein derive from archaeal and bacterial immune systems that evolved to degrade invading nucleic acids [11–15]. In recent years, the continuous development of CRISPR-Cas technology has brought new vitality to diagnostic applications [16–18]. CRISPR-Cas diagnosis systems using Cas effectors (Cas9, Cas12, Cas13, and Cas14) have now been established for detecting various targets [19,20]. Most CRISPR diagnostic techniques rely on **isothermal amplification** technology to detect pathogens even under non-amplification conditions. Such technology enables a faster detection speed with CRISPR diagnostic methods compared with traditional PCR. The accurate recognition of the target sequence and collateral activity of CRISPR proteins that can couple the detection reaction and readout process also ensure a high level of sensitivity of the CRISPR system [21]. The subsequent amplification of detection signals by tool enzymes (e.g., Csm6) [22], the use of a gRNA strategy in tandem [23], and the introduction of a microfluidic system [24] push the sensitivity of CRISPR systems to a level similar to that of PCR. Furthermore, CRISPR detection methods do not require complicated equipment, given that they are essentially a mixed reaction between reagents and samples under general conditions; this makes the operation of the CRISPR diagnostic technology simpler, cheaper, and more portable compared with PCR.

The outbreak of SARS-CoV-2 significantly impacted traditional diagnostic technologies, forcing the development of novel diagnosis technologies. CRISPR technology has moved from being

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an approach used to aid disease diagnosis by PCR to becoming a core technology of the diagnosis system, from diagnosing one pathogen to diagnosing hundreds of pathogens simultaneously, and from detecting nucleic acids of pathogens to detecting a variety of important biomarkers and **SNPs**. Future ideal diagnosis methods are likely to be as simple, as fast, as portable, and as cheap as CRISPR-Cas-mediated diagnosis. Thus, in this review, we discuss the clinical applications and detection limits of various CRISPR diagnostic techniques, the advantages of CRISPR diagnostics in different fields, and the core components of most CRISPR diagnostic systems; we also highlight the developmental prospects of CRISPR diagnostics.

Current CRISPR strategies for nucleic acid detection

Cas9-based detection system

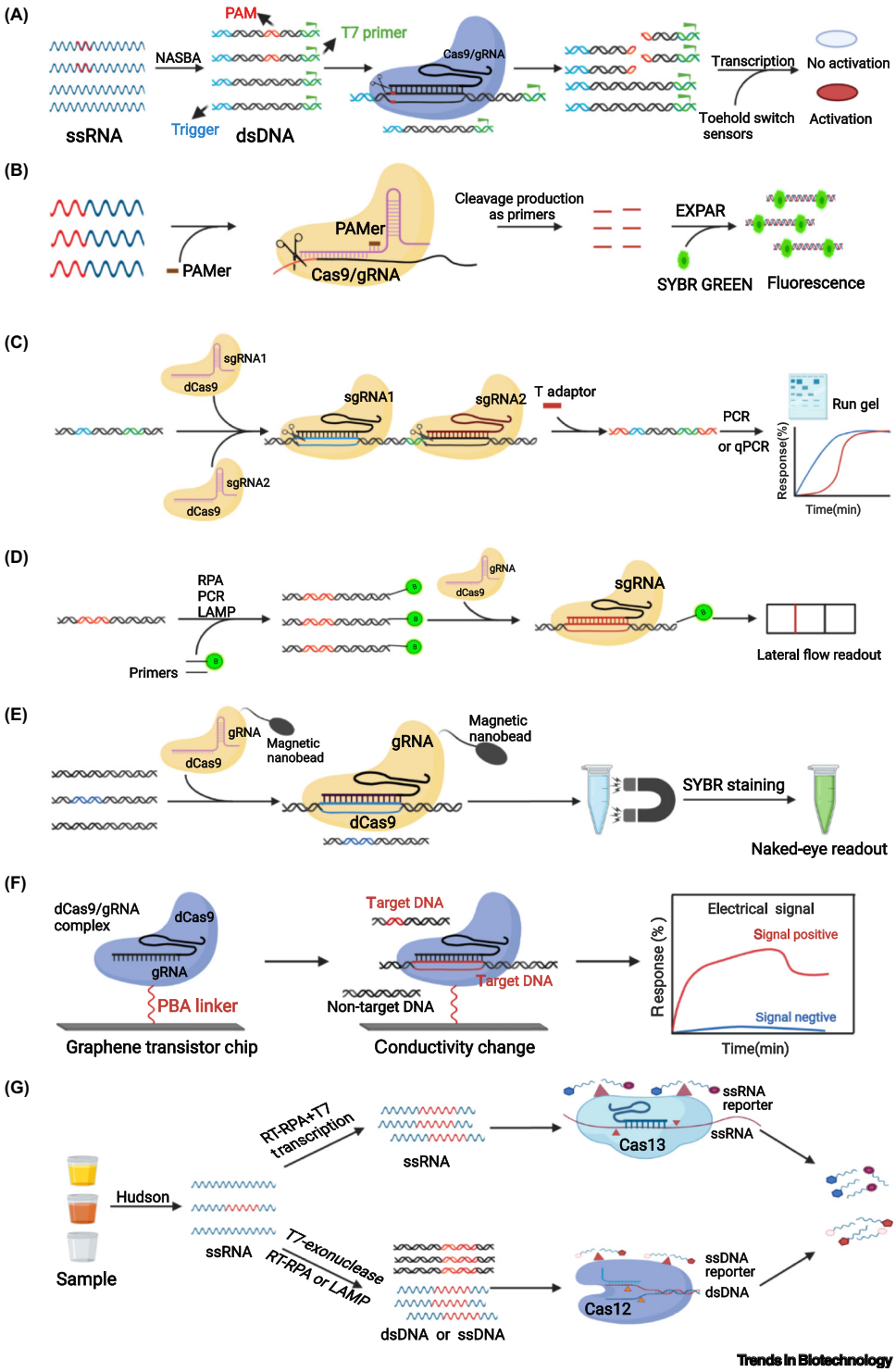
CRISPR-Cas9 is widely applied in the mammalian gene-editing toolbox, including for gene regulation, genetic screening, epigenetic engineering, and genome imaging [25]. Moreover, Cas9 is useful for generating new nucleic acid detection systems [26]. For instance, Pardee and colleagues combined isothermal amplification [**nucleic acid sequence-based amplification (NASBA)**] with Cas9 cleavage activity to detect Zika virus (ZIKV) and used toehold switch sensors to detect the results (Figure 1A) [27]. The high specificity of CRISPR-Cas technology enables it to distinguish between American and African ZIKV strains at a single-base resolution [26,27].

Recently, another research team combined CRISPR-Cas with isothermal exponential amplification to create a new strategy called the CRISPR-Cas9-triggered isothermal exponential amplification reaction (CAS-EXPAR) (Figure 1B) [28]. The DNA fragment produced by Cas9-specific cleavage of targeted DNA activates CAS-EXPAR, generating numerous DNA copies, which can be detected by real-time SYBR Green fluorescence signals [28]. The Cas9 protein itself can also mediate isothermal amplification for nucleic acid detection. Wang and colleagues developed a Cas9 nickase-based amplification reaction system (Cas9nAR) to amplify target sequences at 37°C [29]. This method uses Cas9 nickase to target two sites on one side of the target sequence to release a single-stranded (ss)DNA fragment, which then enters a cycle of extension, cleavage, and replacement by two cleverly designed primers: continuous cleavage by Cas9 nickase and a strand-replacement DNA polymerase. The cyclic amplification process can be quantitatively analyzed by SYBR Green I. The reaction system of this method is simple, low cost, and quick (within 1 h), with a detection limit of 2 copies/20 ml reaction system. Cas9nAR was also combined with lateral chromatography test paper to develop a more convenient reading detection method for food monitoring [30]. In addition, CRISPR-typing PCR (ctPCR) and CRISPR-associated reverse PCR (CARP) methods were developed by combining CRISPR technology with PCR amplification [31–33]. These assays make use of the targeted cleavage activity of Cas9 protein and the specific amplification of target-sequence PCR to obtain the detection results. Specifically, ctPCR uses two Cas9/gRNA complexes to target a specific sequence, the addition of adaptors to target the sequence, and general-specific primers for nucleic acid detection (Figure 1C) [31–33]. Genes from pathogens, such as *HPV16* and *HPV18L1* of human papillomavirus (HPV), were successfully detected by ctPCR and CARP, confirming the feasibility of these methods [31–33]. Furthermore, Wang and colleagues developed a CRISPR-Cas9-mediated lateral flow nucleic acid assay (CASLFA) to successfully detect *Listeria monocytogenes* and African swine fever virus (ASFV) (Figure 1D) [34].

Nuclease-**dead Cas9 (dCas9)** can also be used for nucleic acid detection. For instance, dCas9 fuses with artificially cut luciferase in half to form a new detection method. When two luciferases followed the dCas9/gRNA complex to the adjacent site of the target sequence, the luciferase function was restored and a fluorescence signal was generated. This method was used to successfully detect the DNA of *Mycobacterium tuberculosis* [35]. In addition, a CRISPR-mediated

Glossary

Circulating tumor DNA (ctDNA): released by dead cancer cells into the bloodstream; contains gene mutations from tumor cells and is an important indicator for the diagnosis of cancer.
Dead Cas9 (dCas9): mutant of Cas9; its endonuclease activity is removed by point mutation. It retains the ability to combine with gRNA and target DNA. It can combine with other enzymes to form a gene-editing tool with specific functions.
DNA hydrogel: 3D polymer network based on DNA; perfectly combines the biological function of DNA with the skeleton structure of a hydrogel, with applications in biosensors, cell cultures, drug delivery, protein synthesis, and environmental protection.
Guide RNA (gRNA): synthetic RNA molecule that guides the binding and cleavage of Cas protein to its target DNA sequence by base complementarity.
Isothermal amplification: the isothermal amplification reaction process is always maintained at a constant temperature; rapid amplification of nucleic acid is achieved by adding different active enzymes and their specific primers.
Limit of detection (LOD): usually defined as the lowest concentration or quantity of an ingredient or substance that can be reliably identified by a specific detection method. The LOD of the same detection method will also change with changes in detection conditions; thus, it often fluctuates within a certain range.
Loop-mediated isothermal amplification (LAMP): four specific primers are designed for six regions of the target gene. Under the action of chain replacement DNA polymerase (Bst DNA polymerase), gene templates, primers, and chain replacement DNA synthetases are amplified at 65°C.
miRNA: a type of small noncoding RNAs composed of 20–22 bp that regulate various cellular processes by targeting 3′ untranslated region.
Nucleic acid sequence-based amplification (NASBA): isothermal amplification technique with ssRNA as the template that is mediated by a pair of specific primers and catalyzed by three enzymes.
Protospacer adjacent motif (PAM): short but important DNA motif near the binding site of sgRNA that assists Cas protein in cleaving DNA.



Recombinase polymerase amplification (RPA): protein–DNA complex formed by the combination of recombinant enzymes and primers that can find homologous sequences in dsDNA. Once the primer locates the homologous sequence, a chain exchange reaction occurs, DNA synthesis is initiated, and the target region on the template is amplified.

SNP: polymorphism caused by a single-nucleotide variation at the same site on a genomic DNA sequence between individuals. Research shows that SNP mutations significantly impact human health and are closely related to the occurrence of cancer.

Figure 1. CRISPR-Cas nucleic acid detection technology. (A) Nucleic acid sequence-based amplification (NASBA) detection flow chart. The trigger sequence and T7 primers are added to the RNA fragment amplified by NASBA by reverse transcription. Cas9 cleaves the sequence specifically with protospacer adjacent motif (PAM) to produce different

(Figure legend continued at the bottom of the next page.)

DNA-fluorescence *in situ* hybridization (FISH) flow chart was developed for pathogen detection (Figure 1E). In this method, targeted DNA was isolated from samples by using dCas9-magnetic nanobead complexes and stained with SYBR Green I to obtain naked-eye readouts, which can detect methicillin-resistant *Staphylococcus aureus* (MRSA) at a detection limit of 10 colony-forming units (CFU)/ml within 30 min [36]. By contrast, Hajian *et al.* developed an amplification-free CRISPR chip technology (Figure 1F) [37], the principle of which is to combine dCas9 with electronic transistors made of graphene. The dCas9/gRNA complex can target a particular DNA and connect it with the transistor, thereby changing the conductivity of the latter to produce a detection signal. The graphene transistor used in CRISPR chips has high sensitivity and can detect target DNA as low as 1.7 fM within 15 min. This portable, amplification-free method was used to diagnose exon mutations in clinical samples of Duchenne muscular dystrophy.

In the Cas9 detection platform, Cas9 protein is always used as an independent element to recognize, adhere to, or cut the target sequence, but does not participate in the whole detection process because it cannot easily indicate the result of the reaction without the support of other technologies. Therefore, the Cas9 system is more suitable as a probe that can be combined with a variety of novel nucleic acid detection technologies.

Cas12-based detection systems

Cas12a (also called Cpf1) is a type V CRISPR-Cas protein that contains an RuvC endonuclease domain for DNA cleavage (Figure 1G), including targeted double-stranded (ds)DNA and *trans*-cleaved collateral ssDNA [38,39]. Moreover, a Cas12/gRNA/targeted DNA ternary complex is required for the collateral cleavage activity, which has been exploited in nucleic acid detection platforms, such as the one-hour low-cost multipurpose highly efficient system (HOLMES) and the DNA endonuclease-targeted CRISPR *trans*-reporter (DETECTR) system (Figure 1G) [39,40].

In the HOLMES and DETECTR detection systems, targeted DNA is amplified under isothermal conditions by **recombinase polymerase amplification (RPA)** or reverse transcription **loop-mediated isothermal amplification** (RT-**LAMP**). When it is recognized and bound by the Cas12a/gRNA complex, it stimulates collateral activity and nonspecifically cleaves the DNA reporter to produce fluorescent signals (Figure 2A). DETECTR can be used to detect virus

double-stranded (ds)DNA fragments, which activate the toe switch sensor by transcription to produce truncated or full-length triggered RNA. (B) CRISPR-Cas9-triggered isothermal exponential amplification reaction (CAS-EXPAR) inspection flow chart. Introducing the synthesized PAMer into the Cas9/guide (g)RNA complex can induce site-specific cleavage of the single-stranded (ss)DNA substrate. The resulting cleavage fragments can be hybridized with EXPAR templates and then amplified to obtain real-time SYBR Green fluorescence signals. (C) Circulating tumor (ct)PCR detection flow chart. CRISPR-typing PCR (ctPCR) combines Cas9 nucleic acid cleavage activity with PCR detection. Only the DNA fragments specifically cut by two Cas9/gRNA complexes and with adaptors added can be amplified by PCR or qPCR with a pair of general-specific primers and displayed by fluorescence readings. (D) CRISPR-Cas9-mediated lateral flow nucleic acid assay (CASLFA) detection flow chart. The CASLFA system is a combination of Cas9 technology and a lateral flow system. When Cas9 recognizes and cuts the target sequence, the corresponding bands appear on the lateral flow platform. (E) CRISPR-mediated DNA-fluorescence *in situ* hybridization (FISH) flow chart. This detection method combines magnetic nanobeads with dead (d)Cas9. Once the dCas9/gRNA complex binds to the targeted nucleic acid, the latter is isolated and the signal obtained by SYBR Green staining. (F) CRISPR chip detection flow chart. The principle of this technique is to combine the dCas protein with a graphene electron transistor. The dCas9 recognizes the targeted DNA and connects it to the transistor, which changes the conductivity of the transistor to generate a signal. (G) In the absence of targeted nucleic acids, the collateral activity of Cas protein is not activated and does not cleave the reporter molecule. The nucleic acid in the sample is quickly processed by heating unextracted diagnostic samples to obliterate nucleases (Hudson) technology, and the qualified target sequence (ssRNA for Cas13, ssDNA or dsDNA for Cas12, and ssDNA for Cas12f) is obtained by different amplification techniques [reverse transcription-recombinase polymerase amplification (RT-RPA) and loop-mediated isothermal amplification (LAMP)] and transcription methods (T7 transcription). After binding to its target sequence, the Cas protein activates collateral activity and cleaves the reporter molecule to produce detection signals. These signals can be visually analyzed by fluorescence detection and a lateral flow system.

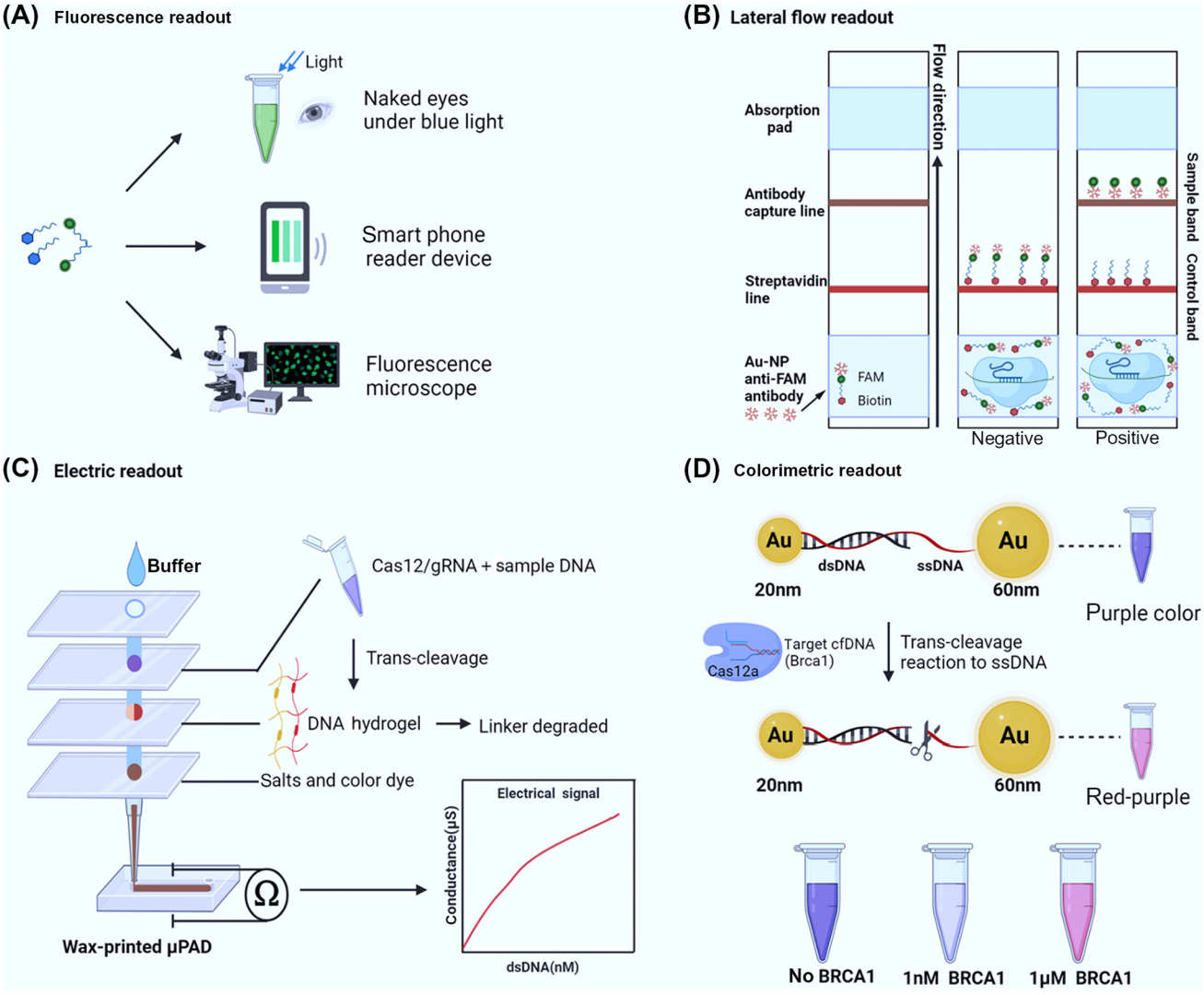


Figure 2. CRISPR detection method readouts. (A) Fluorescence readouts depend on nucleic acid reporter degradation to produce signals. Fluorescence signals can be read by electron microscopes, smartphones, and even the naked eye under blue light. (B) Flow chart of a CRISPR lateral flow detection system. In this systematic reaction process, the Cas protein cleaves FAM-biotin reporter molecules through lateral activity, a reaction that is reflected on the lateral flow test strip. FAM-biotin signal molecules are anchored on the first line (red) of the test strip by anti-FAM antibody–gold nanoparticles (AuNPs), but not on the second line (brown). However, when the reporter is cut, it generates a signal on the second line. (C) Electric readout. DNA hydrogels seal the porous channels of paper-based microfluidic devices, thus hindering the flow of buffer. Detection methods based on DNA hydrogel are used to detect the change in electrical conductivity caused by collateral activity to convert biological signals into electrical signals. (D) Colorimetric readout. Due to the surface plasma effect of AuNPs, increases in the distance between two AuNPs caused by degradation of single-strand (ss)DNA make the solution change from purple to red-purple. Therefore, the concentration of target DNA can be evaluated directly by colorimetry.

genotypes, such as HPV [39,41], whereas HOLMES can detect DNA/RNA viruses and human SNPs within 1 h with an attomolar sensitivity. Interestingly, HOLMES was further developed to form a CRISPR-Cas12a-based method (Cas12aVDet), resulting in HOLMESv2 systems. Cas12aVDet combines the nonspecific cleavage of Cas12a with a ssDNA reporter and RPA amplification in one reaction, reducing the analysis time to 30 min. The fluorescence signal produced by the break of ssDNA can be observed by the naked eye under blue light (Figure 2A) [42]. HOLMESv2 uses a new Cas12b enzyme, which has higher enzyme activity against dsDNA

compared with Cas12a; in addition, it is also more heat resistant than Cas12a [43]. HOLMESv2 combines a Cas12b/gRNA complex with LAMP amplification to detect viral RNA, human cell mRNA, and DNA methylation simultaneously [44]. Furthermore, Joung and colleagues developed the Sherlock Testing in One Pot (STOP) Covid technology by introducing improved gRNA, taurine to improve the reaction kinetics, and flow reading to optimize the one-pot detection method by combining Cas12b and LAMP [45]. In addition, Cas12a was combined with electrochemical sensors to produce the E-CRISPR system. In this system, Cas12a can cleave methylene blue electrochemically labeled ssDNA reporters to produce electrochemical signals that can be detected at the picomolar level [46]. Similar electrochemical signal detection methods also use hairpin DNA (hpDNA) as a reporter molecule. Compared with ordinary ssDNA reporter molecules, hpDNA is easier to react with Cas12a, significantly improving the sensitivity of detection [47]. Cas12 can also bind to DNA-responsive materials to provide new approaches for medical diagnosis. For example, the collateral activity of Cas12 can uncharacteristically degrade ssDNA in the **DNA hydrogel** and change the properties of the latter to produce detection signals [48,49]. This technique can be combined with multilayered paper-based microfluidic devices (μPMDs) to detect pathogen DNA (Figure 2C). The presence of target DNA activates the collateral activity of Cas12 protease and destroys the DNA connectors in the hydrogel, which prevents hydrogel crosslinking and leads to buffer outflow, which is visible to the naked eye [48]. In addition, the system has electrical readouts. The final layer of the microfluidic channel is connected to an ohmmeter to detect electrical conductivity, which depends on the electrolyte provided by the buffer and the amount of outflow (Figure 2C). This readout successfully reduces the detection limit to the sub-nanomole level.

A new type V CRISPR-Cas system, CRISPR-Cas12f (also called Cas14), was recently discovered. Cas12f is a small protein, only half the size of other Cas proteins, and is divided into three subtypes (Cas14a–c) [50,51]. When Cas12f binds to targeted ssDNA, it stimulates collateral activity and cleaves nearby ssDNA nonspecifically, similar to other Cas12 proteins (Figure 1G) [39,50]. Cas12f is not restricted by the **protospacer adjacent motif (PAM)**, which rendered it advantageous in the construction of a new DETECTR system, Cas12f-DETECTR [50]. Interestingly, compared with other Cas12-DETECTR systems, Cas12f-DETECTR shows stronger specificity and activity with allowing high-fidelity detection of DNA SNPs [50,51]. Therefore, the continuous improvement of the Cas12f-DETECTR method enables its use not only for nucleic acid detection of ssDNA pathogens, but also as a high-fidelity and reliable tool for SNP genotyping (such as variants of human *HERC2*, which is responsible for eye color) without PAM constraints [50–52]. Recently, Wu and colleagues have systematically expounded the DNA recognition and cleavage mechanism of small AsCas12f1 nucleases comprising 422 amino acids, which provides a theoretical basis for the development of subsequent molecular diagnostic techniques [53].

The Cas12 detection platform is more suitable for the rapid detection of DNA viruses because the Cas12/CRISPR RNA (crRNA) complex directly recognizes and binds DNA to induce collateral activity. However, other Cas13-based detection platforms require DNA transcription into RNA, which increases the reaction process. Nevertheless, some Cas12 proteins show excellent specificity and are suitable for the detection of SNPs, such as Cas12f.

Cas13-based detection systems

CRISPR-Cas13a (formerly C2c2), a type VI CRISPR-Cas13 effector, contains an RNA-guided ribonuclease that targets ssRNA to protect bacteria from bacteriophage invasion [54]. Combining it with complementary ssRNA activates its specific and nonspecific RNase activity [55,56], the latter causing simultaneous degradation of ssRNA (collateral activity) (Figure 1G). This collateral activity lays the foundation of the specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) method (Figure 1G) [57]. Similar to HOLMES, SHERLOCK also generates signals by degradation

of ssRNA reporters [52]. Based on this theory, a diagnostic test was developed to detect the RNA of specific strains of ZIKV and dengue virus (DENV) [57]. The development of the heating unextracted diagnostic samples to obliterate nucleases (Hudson) method enables SHERLOCK to detect pathogens directly from biological fluids at very low concentrations (as low as 1 copy/μl) [58]. The improved Hudson method inactivates viral hemorrhagic fever (VHF) viruses to avoid the complex extraction process and increase the detection efficiency [59]. RPA or reverse transcriptase RPA can be applied to amplify target DNA, which further improves the sensitivity of SHERLOCK. Moreover, a variety of reagents in the SHERLOCK system can be stored for a long time, which makes this a convenient method to use in the field [57].

In addition, SHERLOCK is being constantly optimized, resulting in SHERLOCKv2 [22]. By studying the specific cleavage preferences of Cas13, SHERLOCKv2 can perform multiple nucleic acid detection on at least four different targets in one reaction. The sensitivity of SHERLOCKv2 was further improved by using an auxiliary CRISPR-related enzyme, Csm6, to amplify the signal of Cas13 collateral activity. Furthermore, combining Csm6 with multiple crRNA-guided Cas13 proteins (multiple crRNA tandem reactions can cover most variants of a pathogen to improve the detection sensitivity) can directly and quickly detect biological samples without preamplification steps. This rapid, amplification-free detection method is called the fast integrated nuclease detection in tandem (FIND-IT) approach [23].

To make CRISPR biosensing platforms easy to carry and use, a lateral flow system for rapid detection was designed by using FAM-BIOTIN reporters [22]. In a lateral flow system, RNA or DNA (depending on the Cas effector) containing fluorescein and biotin at one end is used as a reporter molecule. One end of the reporter molecule (biotin end) is attached to streptavidin, whereas the other (fluorescein) end of the reporter molecule binds to anti-fluorescein antibodies labeled with gold nanoparticle (AuNP)-labeled antibodies (Figure 2B). Once Cas collateral activity is activated by the target sequence, the RNA reporter is cleaved, and AuNP anti-FAM antibodies flow to the test strip. Given that the test band contains secondary antibodies, AuNP-labeled antibodies anchor to the test band and produce colored products that indicate the presence of a targeted sequence (Figure 2B). This method was successfully used to detect mutations in ZIKV and DENV ssRNA virus in fluid biopsy samples of patients [22]. In addition, SHERLOCK has been developed for one-pot tests. Such a one-step reaction can effectively reduce pollution and the difficulty of operation and shorten the reaction time. The sensitivity of the one-pot detection method was improved by adjusting pH, using an optimal concentration of primers, adding RNaseH, simplifying RNA extraction steps, using more heat-resistant Cas proteins, or improving reaction kinetics [60–62]. However, the detection sensitivity of these assays differs from that of multistep methods. To solve this problem, a cell-sized reactor can be used to accommodate the catalytic reaction of Cas13a. The reactor increases the local concentration of samples and RNA reporters. Using this method, Tian *et al.* increased the sensitivity of a one-step ultralocalized Cas13a assay by 10 000 times [63]. Physical isolation of components in a single test tube is also a strategy to reduce the equipment required for this approach [64–66].

In addition to optimization of the detection response, readout methods are also constantly updated. For example, an app on a smartphone is used to identify fluorescent reporter molecules and read the results of lateral flow test strips [10,59,62,66,67] (Figure 2A,B). The app can unify the critical value of detection to reduce the procedures and to provide convenience for checking test results.

Compared to Cas12 detection platforms, Cas13 detection platforms have considerable sensitivity, specificity, and detection speed. Moreover, Cas13 does not rely on a PAM sequence to cut the target sequence, resulting in more application space in the Cas13 detection platform. By

contrast, most unmodified Cas12 proteins need PAM sequences to cleave the target sequence. Therefore, the Cas13 detection platform is given priority in case a PAM is lacking from the target sequence [68,69].

CRISPR-mediated strategies for detecting multiple targets

Other multiplexed CRISPR nucleic acid detection systems include combinatorial arrayed reactions for multiplexed evaluation of nucleic acids (CARMEN) combined with the Cas13 detection method (CARMEN-Cas13). CARMEN-Cas13 pairs nanoscale nucleic acid detection reagent droplets with sample droplets amplified by microfluidic technology [70]; the paired mixture and fluorescence color code are then added to fluorous oil for emulsification, and multiple detection reactions are initiated simultaneously via an electric field (Figure 3A). According to experimental results, CARMEN-Cas13 can detect 169 viruses that infect humans or hundreds of samples of one virus simultaneously. For example, a single microfluidic chip was able to detect 400 samples of coronavirus disease 2019 (COVID-19) simultaneously. In addition, this diagnostic technique can identify all subtypes of influenza A virus and multiple drug-resistant variants of HIV [24], with a 93% level of consistency between CARMEM and next-generation sequencing (NGS) for the latter. Thus, CRISPR detection technology can be used for the generalized detection of viruses. In addition, the high-throughput detection rate of CARMEM is reliable and easy to expand the scale. Because each test result is the median of multiple repeated reactions, the results obtained by this method are more reliable. Although this technique takes ~8 h from extracting nucleic acid to obtaining results, the high-throughput detection capability and simultaneous operation of multiple chips can greatly reduce the detection cycle and cost of a single reaction. Compared with the standard SHERLOCK test, CARMEM technology reduces the reagent cost of each test by 300 times, opening a new optimization direction for CRISPR detection technology.

Thus, CARMEM technology and NGS could become complementary methods for pathogen biology research. CARMEM can be used for primary screening of pathogens before sequencing, which would improve the sequencing efficiency and facilitate further study of pathogen evolution. At the same time, the sequence information of pathogens obtained by NGS could also be further optimized for CARMEN. In addition, CARMEM could be used to perform the large-scale detection of animals (animal vectors and hosts) and humans throughout the transmission chain in the epidemic area, which is vital for the comprehensive surveillance and epidemiological research of any epidemic.

Using CRISPR to reform antibody diagnostic applications

Elledge and team recently developed the peptide immobilization by Cas9-mediated self-organization (PICASSO) technology [71]. PICASSO introduces CRISPR technology into antibody diagnosis experiments by fusing a bespoke peptide library with dCas9. The fusion protein is then labeled by the barcoded single guide (sg)RNA. The fusion protein can be automatically assembled into a DNA microarray containing thousands of target DNAs to construct a protein microarray (Figure 3B). This means that specific locations correspond to specific dsDNA and fusion proteins, which enables researchers to quickly identify antibodies in clinical samples or to study various proteins of interest on a large scale [72,73]. Furthermore, the DNA microarrays for PICASSO can be reused after protease treatment, which can effectively reduce detection costs and reflect a higher value to industry. The research team used PICASSO to detect the presence of antibodies that bind to pathogen proteins in the blood of patients recovering from COVID-19. Compared with traditional protein-screening tools, such as bacteriophage and ribosome displays, PICASSO can standardize protein levels and visualize protein binding, which can improve library representation and avoid amplification deviations [74,75].

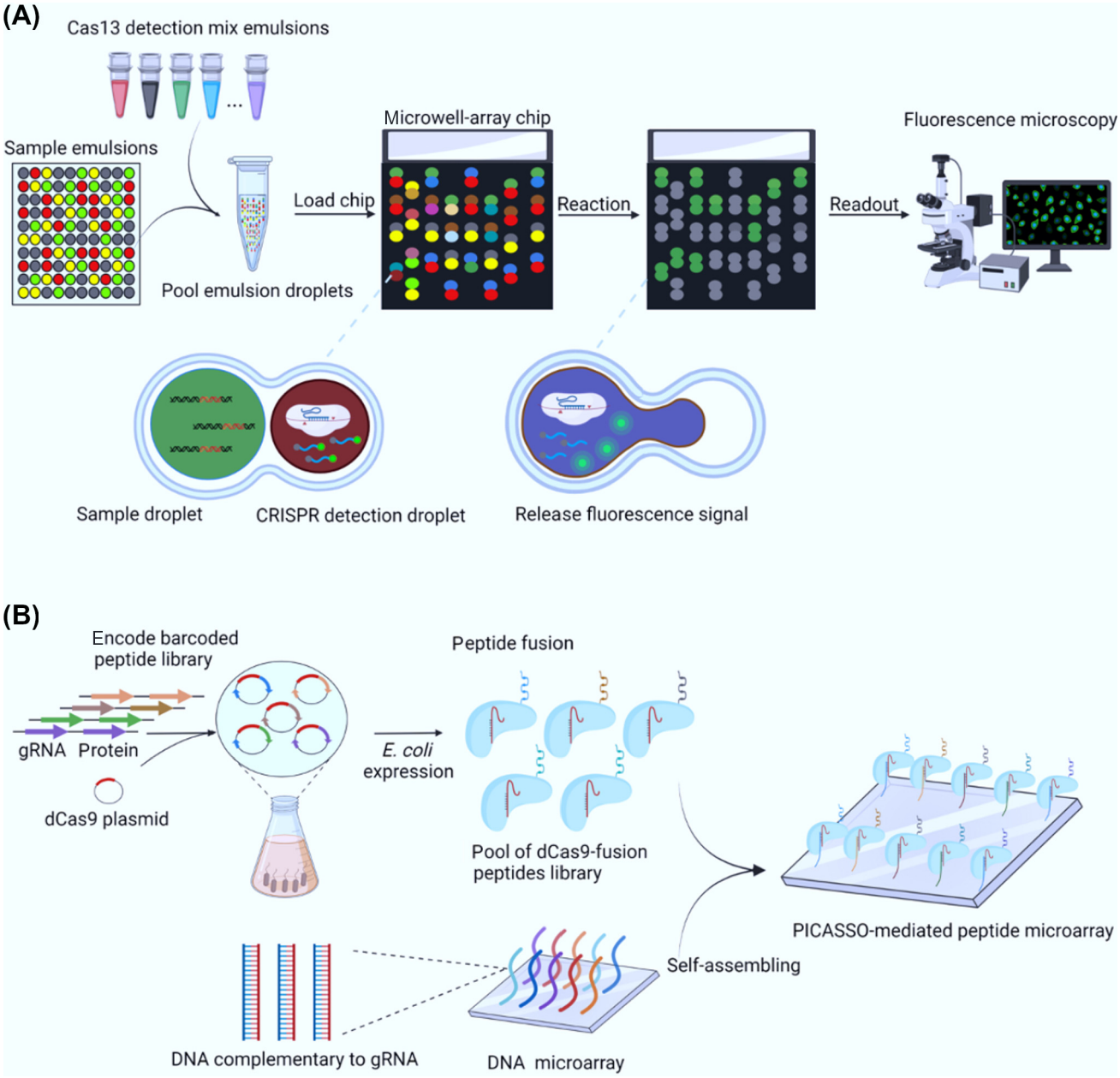


Figure 3. Combinatorial arrayed reactions for multiplexed evaluation of nucleic acids (CARMEN)-Cas13 workflow and peptide immobilization by Cas9-mediated self-organization (PICASSO) technology. (A) In CARMEN, a unique color code is added to the amplified samples or Cas13 detection reagents. The droplets formed by emulsification of the sample and Cas13 detection reagent converge in a single test tube and are then added together to the microwell-array chip to react. Each well on the chip randomly holds two droplets from the sample and the detection reagent, with the reactions in each well being independent of each other. A fluorescence microscope can be used to observe all reactions on the chip. (B) In PICASSO, dead (d)Cas9-fusion peptides self-assemble on the surface of the corresponding DNA microarray. The DNA-templated self-assembling peptide microarrays can be used for protein research and antibody detection.

However, there is also room for improvement of the PICASSO technology. First, PICASSO missed the detection of novel coronavirus antibodies. This can be solved by adjusting the nucleic acid distribution density on the surface of the microarray, reducing background noise, and

analyzing the detection data of more patients to determine a new detection threshold. Second, the length of the peptides used in a PICASSO microarray is less than 40 amino acids. Peptides with a shorter length and incomplete structure might not necessarily represent the epitopes of the corresponding proteins, which limits the study of some proteins with complex structures [71]. It is possible to develop a better plasmid library assembly strategy to display long proteins on PICASSO microarrays. In the future, eukaryotic cell lines could be engineered to express a dCas9-fusion library to accurately display the full-length proteins unique to higher animals. Locating multiple peptides in the same position on the microarray could be used to study and detect multispecific antibodies. Conversely, PICASSO microarrays can also hold numerous antibodies for antigen detection.

Optimization for CRISPR-Cas diagnostics

As mentioned earlier, an ideal nucleic acid detection method would be low cost, simple, specific, fast, portable, and easy to operate [76]. Here, we discuss optimization approaches, current problems, and specific measures for CRISPR diagnosis methods (Table 1).

PCR amplification has always formed the basis of molecular diagnosis technologies. However, its use requires expensive equipment and technical training. Therefore, CRISPR technology is a better choice to meet the demand for a cheap and portable approach. Most CRISPR techniques rely on isothermal amplification technologies, which mainly involve RPA, LAMP, rolling circle amplification (RCA), and NASBA. Of these, RPA and LAMP are the most widely used isothermal amplification strategies for SHERLOCK and DETECTR. RPA-based DETECTR can amplify the target nucleic acid sequence sensitively and rapidly at room temperature (37–42°C), and the primer design of RPA is relatively simple [39]. However, the lower reaction temperature cannot avoid nonspecific amplification and reduces the amplification efficiency due to the combination of primers. At the same time, the mixed enzyme reagents needed for RPA are not fully commercialized. LAMP can improve these problems; a higher reaction temperature (60–65°C) significantly reduces nonspecific amplification, and the amplification efficiency can be increased to more than ten times that of ordinary PCR. The reagents needed for the LAMP reaction are commercially

Table 1. Directions for the optimization of CRISPR-mediated diagnostics

Optimization direction	Current situation	Possible reason	Solution	Refs
Sensitivity/specificity	Sensitivity and specificity of CRISPR diagnostic technology are slightly lower than those of RT-PCR	CRISPR off-target effects Risk of contamination Amplification reaction defects	Optimize Cas enzymes and gRNA Use one-step reaction to reduce contamination Develop improved amplification methods	[112]
Simplicity	Requirements for operators are not low enough	Multistep reaction increases difficulty of operation	Develop a one-pot reaction system with simple sample preparation	[61]
Time	Shortest reaction time is 15 min	Significant room for improvement in sample preparation and amplification	Faster nucleic acid extraction speed or amplification-free extraction strategy	[10,58]
Detection cost	Cost of commercial products is US $30.15/reaction	Reagents used for testing are not fully commercialized	Design a multiplexing detection system to reduce commercialization costs	[24]
Portability	Dependent on nonportable devices	Devices are required to read test results	Use portable readouts, such as lateral flow test strips	[113]
Quantitation	Quantitative ability is generally low	Preamplification of samples increases difficulty of quantitative analysis	Adopt amplification-free strategies or improve amplification technology	[23]

available. In addition, the combination of Cas12b and LAMP led to the concept of a one-pot Cas12 detection platform, which benefited from the optimal matching of Cas12b protein detection reaction and LAMP reaction temperature [44]. However, LAMP primers (four primers) need to cover six target regions, which makes the design process more complicated. As for NASBA and RCA, they also have high sensitivity and specificity, but the high response cost limits their application. NASBA uses three kinds of enzyme (AMV reverse transcriptase, RNaseH, and T7 RNA polymerase) to participate in the reaction, and the reaction costs are high [78]. The RCA method usually requires the design of a padlock probe close to the length of 100 base pairs, which increases the reaction cost [79]. However, the isothermal amplification technique is nonlinear amplification, which makes quantitative analyses difficult [44]. In addition, CRISPR-Cas technology itself has potential off-target effects, which would lead to an increase in false-positive results [80], and may be not easy to fully control after gRNA optimization. The advantages and disadvantages of various CRISPR protein-mediated diagnostic techniques are summarized in Table 2.

Compared to traditional PCR technology, CRISPR technology is fast, cheap, portable, and simple. These advantages are mainly reflected in the one-pot CRISPR-Cas detection method [81]. However, the sensitivity of this method is low and is difficult to optimize. The one-pot reaction is essentially a multistep reaction, and most CRISPR detection systems still need to complete two important steps: the preamplification of the target nucleic acid and identification of the target nucleic acid to stimulate the collateral activity. The first problem is to ensure that the Cas protein maintains high cleavage activity while isothermal amplification is carried out. This often means that the two main reactions need to compromise in the physical and chemical conditions rather than their respective optimal reaction conditions; thus, the sensitivity of the one-pot reaction is not usually high [44]. Second, the extraction of nucleic acid from the sample is a relatively independent experimental step, and the reaction temperature is reduced to room temperature or the isothermal amplification temperature at the expense of detection sensitivity. In addition, some proteases in the nucleic acid extraction kit can inhibit the cleavage activity of the CRISPR system, which is difficult to avoid. The introduction of protease inhibitors to solve this problem will also reduce the efficiency of nucleic acid extraction [45].

Table 2. Advantages and disadvantages of diagnostic strategies

Diagnostic approach	Advantage	Disadvantage	Applicable scope	Refs
Cas9	Compatibility: as most-studied CRISPR protein, it is easily combined with other techniques to derive versatile detection methods Useful for adequate clinical examinations	Technology dependence: always requires special labeling of sample to indicate detection readout	Can be combined with most emerging molecular diagnostic techniques to derive new diagnosis strategies	[114]
Cas12	Simple sample preparation: directly targets dsDNA without other processing of amplified sequence High sensitivity and specificity Cheap: cost of testing is similar to that of SHERLOCK	PAM dependence: PAM required when targeting nucleic acids Optimization difficulties: optimization process of multiple reactions is complex, especially one-step detection method	Especially suitable for DNA sequence and SNP detection	[51,82]
Cas13	Fast: SHERLOCK using RPA technology can detect targeted nucleic acids within 5 min (without RPA time) High specificity: SHERLOCK can distinguish single-nucleotide mismatches in targeted nucleic acid sequences; thus, it is easy to distinguish virus genotypes Cheapness: low cost of SHERLOCK; typical single-chain reaction is estimated to cost US$0.60	Poor quantitative ability: amplification of samples in detection increases difficulty of quantitative analysis Optimization difficulty: optimizing all reactions in detection process requires professional biological knowledge	Not limited by PAM sequence Can be used to target arange of nucleic acid sequences	[81]

Biological and biomedical applications of CRISPR technologies

CRISPR detection for infectious disease

CRISPR technology has high clinical value in the diagnosis of infectious diseases because of its low cost, rapidity, and accuracy. The main pathogens detected by CRISPR technology include viruses, bacteria, and parasites.

The SHERLOCK detection system was developed in response to the current COVID-19 pandemic for immediate novel coronavirus detection. SHERLOCK-mediated diagnostics comprises three initial key reactions: an RT-RPA-mediated preamplification reaction, T7 transcription, and a Cas13 detection reaction (Figure 1G). The assay reached a **limit of detection (LOD)** of 42 SARS-CoV-2 copies/reaction from nasopharyngeal and throat swabs from patients; readouts were 100% specific and only took ~70 min [21]. Furthermore, the optimized SHERLOCK reaction system for SARS-CoV2 detection includes: (i) RT-LAMP technology with higher amplification efficiency to replace RPA [45], (ii) one-step reaction systems [62], and (iii) development of nonamplification detection methods [10] (Table 3). Another Cas13 protein-mediated one-step detection method is streamlined highlighting of infections to navigate epidemics (SHINE) [62]. The improved Hudson method can quickly inactivate the virus in the sample within 10 min, thus speeding up detection. The method was used to detect nasopharyngeal swabs from 50 patients with SARS-CoV-2 within 50 min. This method showed 90% sensitivity and 100% specificity against RT-qPCR. Recently, a new amplification-free detection technique was developed that combines multiple gRNAs targeting multiple sites of the target nucleic acid to increase detection sensitivity, while the results can be read using a mobile microscope. This method can detect SARS-CoV-2 RNA extracted above 100 copies/ul within 30 min and correctly identified five samples of novel coronavirus from patients. The concentration of SARS-CoV-2 RNA in the sample ranged from 2.08×10^{10} to 1.27×10^{8} copies/ml [10].

In addition to the SHERLOCK technology, Cas12-mediated DETECTR-targeted E (membrane protein) gene and N genes (nucleoprotein) were used for detecting SARS-CoV-2 infections. The assay reached an LOD of 10^4 copies/ml and the whole detection process took less than 40 min, with a 95% positive predictive rate for patients with SARS-CoV-2 infection [82]. Joung and coworkers developed STOPCovid technology to detect SARS-CoV-2 within 1 h, with sensitivity close to that of qPCR. They used STOPCovid to examine nasopharyngeal swabs from patients with SARS-CoV-2, correctly diagnosing 12 positive and five negative cases [45]. Moreover, STOPCovid.v2 integrated a RT-LAMP amplification reaction to reliably detect a viral load with an LOD of 2000 copies/ml, with a sensitivity of 93.1% and a specificity of 98.5% for swab samples obtained from patients [61]. In addition, Ding and coworkers developed an all-in-one dual CRISPR-Cas12a (AIOD-CRISPR) detection method, with no restriction by PAM sequences [83]. This avoids the difficulty of detection and the risk of pollution caused by multiple operations. The sensitivity of this method reached five copies of synthetic SARS-CoV-2 RNA, and all eight SARS-CoV-2-positive samples in 28 clinical swab samples were successfully detected. Besides SARS-CoV-2, there are other viruses that can be detected by CRISPR technology (Table 3). For example, Cas13a-mediated SHERLOCK is used to detect opportunistic BK virus (BKV) and giant cell infection after organ transplantation. This assay was used to detect BKV in 36 plasma samples and 31 urine Hudson-treated samples of patients with 100% sensitivity and specificity. Similarly, the sensitivity and specificity of cytomegalovirus (CMV) were 80% and 100%, respectively. The readouts of this approach are that the smartphone software quantifies the strip strength of the lateral flow test strip, which can reduce the influence of background noise and detector deviation on the results [84].

CRISPR diagnostic products approved for clinical use continue to emerge. The Sherlock CRISPR SARS-CoV-2 kit developed by Sherlock Biosciences received emergency use authorization from

Table 3. Use of CRISPR-Cas for disease diagnosis[a]

CRISPR-based method	Pathogen	Name	Preamplification	Assay time[b] (min)	Readout	LOD[c] (cp/ml)	Refs
Cas13	SARS-CoV-2	SHERLOCK	RT-RPA	70	Fluorescence or lateral flow	42 copies/reaction	[21]
		DISCoVER	RT-LAMP	35	Fluorescence	4×10^4	[117]
		SHINE	RT-RPA	50 (one step)	Fluorescence or lateral flow	5×10^3	[62]
		Amplification-free CRISPR-Cas13a assay	Amplification free	30	Fluorescence	10^5	[10]
	Ebola virus	Solution-based CRISPR assay	Amplification free	5	Fluorescence	5.45×10^7	[70]
	DENV; ZIKV; *Pseudomonas aeruginosa*; *Staphylococcus aureus*	SHERLOCK	NASBA or RPA	132 (NASBA) or 120 (RPA) and 60–180 (Cas13)	Fluorescence	1.2×10^3	[57]
		SHERLOCKv2b	RPA	60 (RPA) and 60–180 (Cas13) or 60–180 (one pot)	Fluorescence or lateral flow	2 aM	[22]
	EBV	SHERLOCK	RPA	NS	Fluorescence	8 copies/reaction	[119]
	CMV, BKV	SHERLOCK	RPA	NS	Lateral flow	NS	[84]
	Salmonella enteritidis	APC-Cas	Allosteric probe-relevant amplification	110 (APC) and 30 (Cas13)	Fluorescence	NS	[86]
	Plasmodium spp.	SHERLOCK	RT-RPA	30 (RPA) and 180 (Cas13)	Fluorescence	2.5 parasites/μl	[87]
Cas12	SARS-CoV-2	DETECTR	RT-LAMP	40	Lateral flow	10^4	[82]
		AIOD-CRISPR	RPA	20 (one step)	Naked eye	5 copies/reaction	[83]
		STOPCovid	LAMP	60 (one step)	Fluorescence or lateral flow	2×10^3	[61]
	Pseudorabies virus	HOLMES	PCR	88 (PCR) and 15 (CRISPR)	Fluorescence	6.6×10^3	[40]
	JEV	HOLMESv2	LAMP	40 (LAMP) and 35 (CRISPR) or 120 (one pot)	Fluorescence	6.0×10^3	[44]
	HPV	DETECTR	RPA	10 (RPA) and 60–120 (CRISPR)	Fluorescence	6.0×10^2	[44]
	Mycobacterium tuberculosis	CRISPR-MTB	RPA	30 (RPA) and 20 (Cas12)	Fluorescence	5.0×10^3	[41]
PCR	SARS-CoV-2	Real-Time Fluorescent RT-PCR Kit	RT-qPCR	180	Fluorescence	1.0×10^2	[120]
	Ebola virus	Quanty ZEBOV FAST kit	RT-qPCR	60	Fluorescence	3.74×10^2	[121]
	DENV	RealStar Dengue RT-PCR Kit 3.0	RT-qPCR	120	Fluorescence	7.04×10^3	[122]
	ZIKV	RealStar Zika virus RT-PCR Kit 1.0	RT-qPCR	NS	Fluorescence	6.1×10^2	[123]
	P. aeruginosa	LightCycler FastStart DNA HybProbe kit	RT-qPCR	105	Fluorescence	50 cfu/ml	[124]

(continued on next page)

Table 3. (continued)

CRISPR-based method	Pathogen	Name	Preamplification	Assay time[b] (min)	Readout	LOD[c] (cp/ml)	Refs
	S. aureus	sodA nested assay	RT-qPCR	120	Fluorescence	10 cfu/ml	[125]
	EBV	TaqPath® multiplex PCR master mix	qPCR	72	Fluorescence	1.25×10^2	[126]
	CMV	TaqPath® multiplex PCR master mix	qPCR	72	Fluorescence	1.0×10^2	[126]
	BKV	TaqPath® multiplex PCR master mix	qPCR	72	Fluorescence	1.83×10^2	[126]
	S. enteritidis	QuantiTect Multiplex PCR NoROX Master Mix	qPCR	300	Fluorescence	1.0×10^3	[127]
	Plasmodium spp.	abTES™ Malaria 5 qPCR II kit	qPCR	NA	Fluorescence	0.125 parasites/μl	[128]
	JEV	TaqMan1 Fast Virus 1-Step	RT-qPCR	NA	Fluorescence	1.2×10^2	[129]
	HPV	Digital droplet PCR	qPCR	NA	Fluorescence	80	[130]

[a]Abbreviations: APC-Cas, allosteric probe-initiated catalysis and CRISPR-Cas13a system; BKV, BK virus; CMV, cytomegalovirus; EBV, Epstein–Barr virus; JEV, Japanese encephalitis virus; NA, not applicable; NS, not specified.
[b]Assay time indicates the approximate incubation time most frequently used in the referred study (depends on the corresponding sensitivity, response mode, and readouts).
[c]LODs of various detection methods are not necessarily comparable, because some studies do not clarify how they were obtained. LODs shown here are generally under the optimal reaction conditions of that method; LODs of individual studies cannot be converted into standard units; thus, the description used in the article is adopted. Generally speaking, LOD depends on sample preparation method, amplification method, readouts, and incubation time.

the FDA. This kit detects SARS-CoV-2 nucleic acid in upper respiratory tract samples and bronchoalveolar lavage fluid samples from patients with suspected SARS-CoV-2 within 1 h. Compared to the RT-PCR comparator assay also authorized by the FDA, the sensitivity of the Sherlock CRISPR SARS-CoV-2 kit was 96.7% (29/30) and the specificity was 97.0% (64/66)[i]. Moreover, a thermostable CRISPR-Cas enzyme was discovered that provided a simple multiple diagnostic solution for automation of SHERLOCK testing. In addition, Shanghai BioGerm Medical Technology Company recently developed a HOLMES-based constant temperature nucleic acid amplification and detection analyzer, BG-NOVA-X8, and obtained Chinese National Medical Products Administration (NMPA) certification. BG-NOVA-X8 is a piece of automatic CRISPR detection equipment integrating magnetic beads for efficient nucleic acid extraction, RPA amplification, and HOLMES detection, which can complete analysis of 24 clinical samples in 30 min. The detection limit of BG-NOVA-X8 (800 copies/ml) is close to that of qPCR (100–500 copies/ml)[ii].

In addition to using CRISPR to detect viruses, CRISPR-mediated diagnostics can sense bacteria, including *M. tuberculosis* [41,57], *S. aureus* [22,57], *Pseudomonas aeruginosa,* and *Salmonella enteritidis* [57]. Ai and coworkers developed a Cas12a-mediated diagnostic system for *M. tuberculosis* and validated the method by testing clinical samples from 179 patients with tuberculosis (TB). The results showed that the sensitivity of this method was as high as 79%, which was higher than that of culture (33%) and the GeneXpert MTB/RIF methods (66%) [41]. Sam *et al.* recently developed the Cas12b-based TB-Quick method (TB-QUICK) for sensitive diagnosis of *M. tuberculosis* within 2 h, with a detection limit of 1.3 copies/μl. Its sensitivity for clinical pulmonary TB samples was 86.8%, which was significantly higher than for *M. tuberculosis* culture (66.7%) and Xpert (70.4%) [85]. Zhang and coworkers developed a multiplex

SHERLOCKv2 detection platform based on the specific cleavage preference of various Cas13 enzymes for nucleic acid reporter molecules. This method can simultaneously detect *S. aureus* and *P. aeruginosa* at the attomolar level [22]. Gootenberg and coworkers successfully identified the genotype of gDNA isolated from *P. aeruginosa* by the SHERLOCK technique [57]. Shen and coworkers developed an APC-Cas detection method by combining nucleic acid-based allosteric probes (APs) with CRISPR-Cas13a [86]. In this method, the AP was used to identify *S. enteritidis*. When the aptamer domain of the AP binds to the target bacteria, it catalyzes the AP for DNA sequence extension and transcription, resulting in a large number of ssRNAs entering the Cas13 reaction system to generate detection signals. This technique can quantitatively detect the number of *S. enteritidis* (1–10^5 CFU) in various samples. The cost of the reagents used for testing is as low as US$0.86 per test.

By contrast, SHERLOCK technology has an important role in the detection of *Plasmodium*. Cunningham and coworkers designed a SHERLOCK system to detect all *Plasmodium* species known to cause malaria and another system to detect sulfadimethoxine-resistant *Plasmodium falciparum* [87]. The former was used to detect 123 clinical samples with 94% sensitivity and specificity (compared with fluorescence qPCR), whereas the latter was used to detect 43 clinical samples with 73% sensitivity and 100% specificity (compared with deep sequencing). These results reveal the potential of CRISPR technology for the detection of parasites.

Cancer biomarker detection in the age of CRISPR

Detection of cancer-related biomarkers is of benefit for the prognosis of patients with cancer [88,89]. CRISPR testing technology can detect different types of cancer biomarker [90]. Compared with traditional detection methods, such as PCR and ELISA, CRISPR detection technology does not require expensive instruments and experienced operators [34,91]. For example, CRISPR technology can detect **circulating tumor DNA (ctDNA)** and **miRNA** in human circulation. Cas12a detection systems for EGFR790 and EGFR858 can be used to assess cancer progression [92], detecting EGFR858 and EGFR790 in less than 3 h with a detection limit of 0.005%. A CRISPR/Cas12a-based nonamplification strategy to detect breast cancer gene 1 (*BRCA1*) has also been developed. This uses two partially hybrid ssDNAs to connect two AuNPs of different sizes. The end of one ssDNA is modified by fluorescence and quenched by large-size AuNPs. When the target ctDNA activates Cas12a collateral activity, the unpaired ssDNA is cut off, resulting in the dissociation of the two AuNPs to produce fluorescence signals and a color change of the solution from purple to red-purple (Figure 2D). This method can get results in 30 min, with an LOD of 0.34 fM [93]. The detection of miRNA (let-7a) by a CRISPR-Cas12a system is significant for the early diagnosis of cancer. Unfortunately, the sensitivity of directly using CRISPR technology to detect miRNA is relatively low. At present, the detection limit of PCR-mediated miRNA peaks at 9 fM, but is difficult to achieve [94]. Hyperbranched RCA was used instead of PCR technology to solve this problem, reaching a final LOD of 0.24 zmol [95]. However, the isothermal amplification method often produces nonspecific background signal interference detection results. As a result, Cas13a-mediated direct detection of miRNA has been developed, which can detect target miRNA within 30 min, with a detection limit of 4.5 amol. This method was used to detect the relative quantification of miR-17 in four cell lines, and the results were consistent with those of qRT-PCR [96].

In addition, CRISPR systems can also detect protein biomarkers. Chen and coworkers combined CRISPR technology with ELISA to improve its ability to detect human vascular endothelial growth factor. The detection limit of this method was 32.27 fg/ml, which is ~100 times higher than that of conventional ELISA detection [97]. Dai and coworkers designed corresponding aptamers to interact with, and detect, TGF-β1 [46]. They used Cas12a-mediated electrochemical biosensors to identify aptamers to detect TGF-β1 in samples, with a detection limit of 0.2 nM. Zhang's

team designed a dsDNA–aptamer complex to bind to alpha-fetoprotein (AFP) and activate the collateral activity of the Cas12a system, resulting in the generation of detection signals [98]. On this basis, they developed biosensors for the detection of AFP and cocaine in human serum. The detection limits were 0.07 fmol/l and 0.34 μmol/l, respectively. Zhao and coworkers activated the CRISPR system with the help of a polynucleotide kinase (PNK) catalytic ligation reaction [99]. Protein detection is mostly based on the characteristics of the protein itself or of specially designed aptamers, which will limit the application of the CRISPR system in this field.

Extracellular vesicles contain nucleic acids, proteins, and lipids, which reflect the state of the parent cell [100]. Among them, the proteins and nucleic acids in EVs have broad prospects in cancer diagnosis [101]. The CRISPR technique can detect proteins in exosomes [102,103] in the same way as for miRNA in the exosome, via the combination of a CRISPR/Cas12a system and rolling cycle amplification [104].

CRISPR-based SNP genotyping method

There are many methods available to detect SNPs, such as pyrophosphate sequencing [105], restriction fragment length polymorphism (RFLP) PCR, fluorescence qPCR (TaqMan PCR) [106], and mass array methods. However, these methods have issues, such as expensive equipment, low accuracy, need for highly trained personnel, and so on. The high sensitivity, high specificity, portability, and simplicity of CRISPR detection platforms can overcome these issues. The SHERLOCK system has been used to distinguish between ZIKV and DENV subtypes [58]. The Cas14a-based DETECTR platform was also used to identify single-nucleotide mutations in *HERC2* [50]. However, these two techniques themselves cannot directly target dsDNA, which would add additional reaction steps. To do so, a Cas12b-mediated DNA detection platform (CDetection) can be used to directly identify dsDNA. This increases the signal-to-noise ratio by removing complementary ssDNA and artificially increasing nucleotide mismatches, which improves the resolution of the method to better detect heterozygous mutations [107]. In addition, Cas12b-based HOLMESv2 used asymmetric PCR and LAMP amplification methods to detect the rs5028 SNP in HEK293T [44]. At the same time, to make the detection more automated and reduce bias, the researchers also introduced microfluidic biochip technology [108], confirming the accuracy of their CRISPR-Cas12a SNP detection chip by detecting the SNPs CYP1A1*2 (A4889G, rs1048943), CYP2C19*2 (G681A, rs4244285), CYP2C9*3 (A1075C, rs1057910), and CYP2C19*3 (G636A, rs4986893) and comparing their results with those of pyrophosphate sequencing.

Concluding remarks

To use CRISPR diagnosis technologies for the clinical diagnosis of infectious diseases, such as SARS-CoV-2, the automation of CRISPR diagnosis technology is almost an inevitable requirement (Figure 4). Such automation of detection means reducing operating difficulties and labor costs, speeding up the testing speed, and unifying the diagnostic criteria [109], which not only expands the original advantages of CRISPR diagnostic technologies, but is also in line with the standards of ideal diagnostic methods [76]. In addition, the point-of-care (POC) diagnosis field has always been the main clinical-oriented approach for CRISPR diagnosis. Reviewing the development of POC diagnosis over the past few years has shown that integrating the full detection process into a fully automated single device is both a challenge and an opportunity, which is necessary for the commercialization and wide application of these detection methods [110,111]. The main aim is to integrate sample processing, preamplification, CRISPR detection, and reading process of the CRISPR diagnosis system into a single device (Figure 4). In terms of the CRISPR diagnosis system itself, sample processing is gradually being simplified, nonamplification strategies are being devised, and result reading methods are already fully developed, which could lead to the development of a fully integrated CRISPR detection device in the

Outstanding questions

Can a CRISPR system be developed that is small enough in size and catalytic activity for nucleic acid detection to differentiate among different types of bacteria and archaea?

Can isothermal amplification be further optimized to improve the quantitative ability of CRISPR detection?

Can we improve the specificity of detection by regulating the activity of Cas proteins by small molecules without interfering with the normal reaction?

How different will test results from different operators and different quality biological samples be? Might they be prone to missed diagnoses or misdiagnoses?

How can the perfect integration of each component in the future fully automatic integrated CRISPR detector be realized?

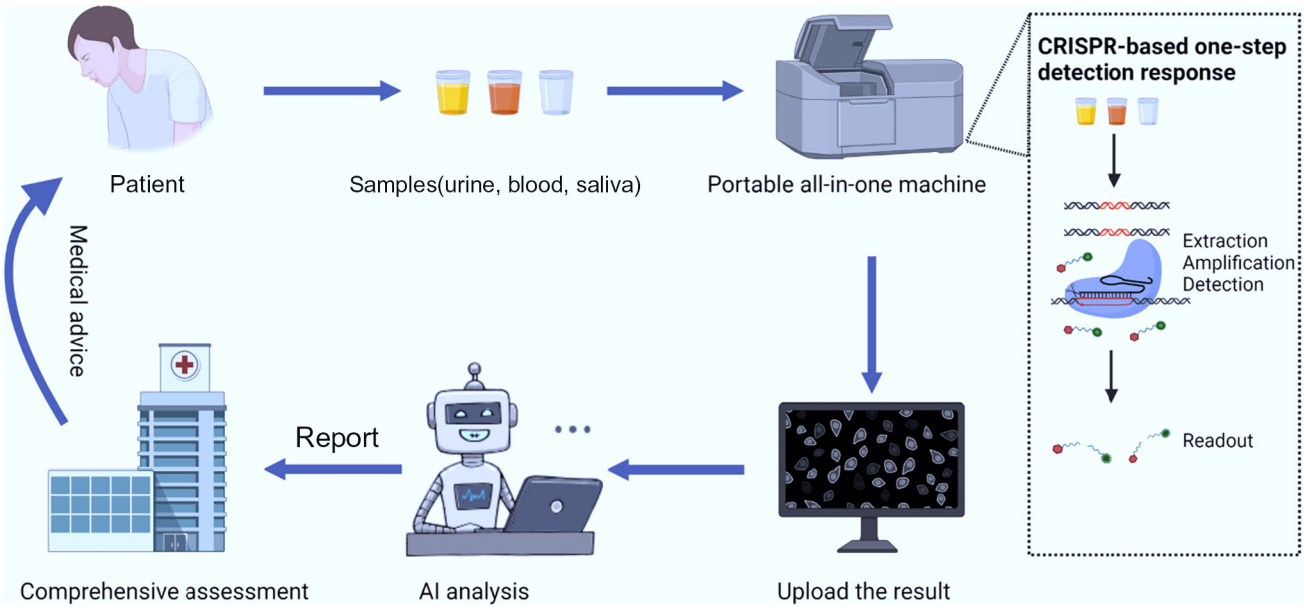


Figure 4. The future of CRISPR/Cas rapid detection systems. Samples from patients in community and medical sites can be tested cheaply and quickly through portable and integrated testing equipment. The test results would be uploaded through the network, screened by artificial intelligence (AI), and finally sent to the hospital, where doctors will be able to provide medical guidance to the patients according to the results.

near future. The optimal example so far is the BG-NOVA-X8 all-in-one detector recently developed by the Shanghai BioGerm Medical Technology Company. Therefore, the integrated components include not only the completion of the response, but also the digital display of the results and even the online upload of the diagnostic results to link up with the required treatment process. The construction of a complete diagnostic and treatment network significantly improves the level of diagnosis and resulting treatment decisions (Figure 4). Achieving this level of detection requires the optimization of all reactions, the development of highly efficient biosensors, and the introduction of artificial intelligence (Figure 4) [80].

CRISPR diagnostic technologies still face many challenges (see Outstanding questions). For instance, they might rely too much on isothermal amplification technology, in which the number of nonamplification diagnosis methods and sensitivity are both relatively low. However, the nonamplification method can greatly reduce the detection time and avoid the interference of isothermal amplification on the quantitative analysis, which is an important route for further optimization of CRISPR molecular diagnosis technologies. And the off-target effect of Cas protein will also fundamentally affect the LOD of the whole system. Regulating the enzyme activity of CRISPR protein to reduce off-target effect is a prominent problem at present. CARMEN, based on a microfluidic system, shows a significant advantage of multiplexed diagnosis. However, it is still difficult to develop a simple single-tube multiplexed CRISPR diagnosis method that does not rely on expensive equipment. A fully automatic detection technology requires the coordination of four components (sample processing, amplification mode, CRISPR component, and result reading), which is currently lacking but could become a reality in the near future.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Resources

[i]https://sherlock.bio/partnerships/
[ii]http://bio-germ.com/

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