The emerging improved approaches for CRISPR/Cas-based detection: Recent advance and perspectives

Ziyue Liang[1], Xinran Xiang[2*]

[1]College of Agriculture, Henan University of Science and Technology, Luoyang, 471023, China

[2]South China University of Technology, Guangzhou, China

[*]corresponding author: Xinran Xiang

Abstract

The rapid spread of infectious and non-infectious diseases highlights the critical need for precise, affordable, and field-deployable diagnostic tools, especially in resource-constrained settings. Traditional methods, while widely used, often fall short in meeting the required sensitivity, specificity, and operational efficiency. The CRISPR-Cas system, originally an adaptive immune mechanism in bacteria, has been repurposed into a powerful biosensing tool, demonstrating exceptional potential for accurate and versatile detection. This review examines the current limitations of conventional diagnostic techniques and explores the advancements in CRISPR-Cas‐based biosensing. Innovations such as the integration of CRISPR-Cas with amplification methods, nanomaterials, and microfluidic platforms have significantly enhanced its sensitivity, speed, and portability. Challenges like enzyme stability, anti-CRISPR proteins, and PAM dependency remain but are being actively addressed. In summary, CRISPR-Cas systems, combined with other cutting-edge technologies, hold immense promise for providing customized and efficient detection methods, paving the way for revolutionary

improvements in diagnostics across diverse applications.

1. Introduction: Recently, with the outbreak of COVID-19 and the epidemic of diseases such as monkeypox, highly sensitive and rapid detection is crucial to facilitate the allocations of medical resources effectively in the hospitals. Especially for low-income countries, rapid and affordable on-site and point-of-care diagnostics has a significant impact on detection of infectious and non-infectious diseases that may have weak health systems and limited access to medical services. Therefore, swift, convenient, cost-effective and feasible detection methods for large-scale screening, on-site detection and point-of-care diagnosis are of great significance and urgency for the rapid control of the fast spread of the virus and the rapid diagnosis of other diseases.

CRISPR/Cas is a vital component of the bacterial adaptive immune system, which can employ CRISPR RNA (crRNA)-guided nucleases to prevent prokaryotic cells from foreign nucleic acid invasion[1].The emergence of CRISPR/Cas detection system has significantly altered the landscape of detection management, including tumor screening and diagnosis, foodborne pathogen detection, animal and plant research and treatment of non-infectious diseases. The major goal of CRISPR/Cas detection system is to achieve accurate and highly sensitive targeting. Traditional molecular diagnostic techniques of clinical samples and food environment are generally achieved with special genes obtained by polymerase chain reaction (PCR) and nucleic acid hybridization technique. However, there are several limitations associated with detection, such as trained personnel restrict, the required laboratory infrastructures, poor associativity to target analytes, and difficulties in avoiding false positive problem.

Unlike routine test methods, CRISPR/Cas-based detection is an ultrasensitive technique to detect disease or food samples, such as virus, leukemia, and single cells. This tool offers the opportunity to monitor pathogene progression and achieve efficient knockout of specific genes. CRISPR/Cas system can also be applied to help in new drug development and small molecule activity identification. Consequently, CRISPR/Cas system is considered one of the most innovative techniques and is opening previously unexpected prospects.

The unique characteristics of CRISPR/Cas system have attracted extensive research interest in detection. However, CRISPR/Cas detection system exists some limitations and disadvantages, which imposes restrictions on its application scopes. For example, Cas enzymes are discrepant and unstable. They are gradually unable to keep pace with the requirements of rapid detection, which requires ultra-high cleavage efficiency, for example, the expression level of fluorescence signal is usually low but has great relationship in the presentation of results. Therefore, the improvement of CRISPR/Cas detection system is urgently needed. The interest in CRISPR/Cas-based assays or bioimaging is increasing because of the improvement of CRISPR/Cas system elements, the innovative design and more strong instruments. Those advancements, which improve the sensitivity, precision and velocity of CRISPR/Cas-based assays, have motivated the attempts to meet the ongoing need of the detection. Consequently, in this review, we will be focus on improved measures and principles of the CRISPR/Cas system itself. Additionally, based on CRISPR/Cas detection system, we proceed to depict signal enhancement on nanomaterials, versatile integration and various

amplification strategies for nucleic acid detection. We also describe a large range of CRISPR/Cas-based detection biosensors for signal output, including optical, electrochemical, etc. We come to an end with a discussion of future possibilities and challenges in the field of CRISPR/Cas-based detection. We are committed to providing ideas and inspiration for the further development and improvement of CRISPR/Cas in the future.

2. The fundamental principles of CRISPR/Cas systems

2.1 Fundamental components of CRISPR/Cas systems and their discrepancies

The CRISPR/Cas system consists of two major parts: The first segment is the gene encoding Cas-related proteins in the upstream. These proteins encoded by this gene can interact with the CRISPR sequence region, so the gene is named the Cas gene. The left part is called CRISPR array comprising repeat sequence and spacer. In addition, the leader that is in the upstream is regarded as the promoter of CRISPR sequences (Figure 1A). CRISPR/Cas system depends on guide RNA to recognize its complementary DNA or RNA targets. The Cas enzyme is activated to cleave them into debris once it succeeds to recognize targets. And the result will display fluorescence signals at the present of fluorescent probe (Figure 1B).

However, distinct effector proteins have different functions because of structural discrepancies (Figure 2). Cas9 is a single RNA-guided double stranded DNA (dsDNA) effector protein with CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA). Cas9 recognizes the target with a protospacer adjacent motif (PAM) sequence restriction on target DNA and cut the dsDNA using its RuvC and His-Asn-His (HNH)

domain to product dsDNA strands. The binding ability of another Cas9 effector, dCas9, is the same as Cas9, while there is no endonuclease activity[2]. Correspondingly, Cas12a/b identify dsDNA by crRNA and cleave target strands with the guidance of PAM as well. But they only have one endonuclease domain-RuvC**.** After finishing the cleavage, the collateral cleavage of untargeted sequences (trans-cleavage) of Cas12a/b is triggered and set about to cut single-stranded DNA (ssDNA)[3]. Unlike Cas9 and Cas12, Cas13a combines with this mature crRNA and forms cleavage sites on the target RNA using Two identical higher eukaryotes and prokaryotes nuceotide-binding (HEPN) domains. The cleavage of Cas13a does not demand a PAM, but a required sequence motif adjacent to the target site, termed the protospacer flanking site (PFS). Also, Cas13a can generate trans-cleavage activity and cleave non-target ssRNA indiscriminately[4]. The Cas14a is a ssDNA nuclease which is recently discovered. It can not only combine with target ssDNA via crRNA, but also bind to non-specific ssDNA. Cas14a showed the capacity of recognition and cleavage of ssDNA sequences with single Ruvc domain independent of a PAM or PFS.

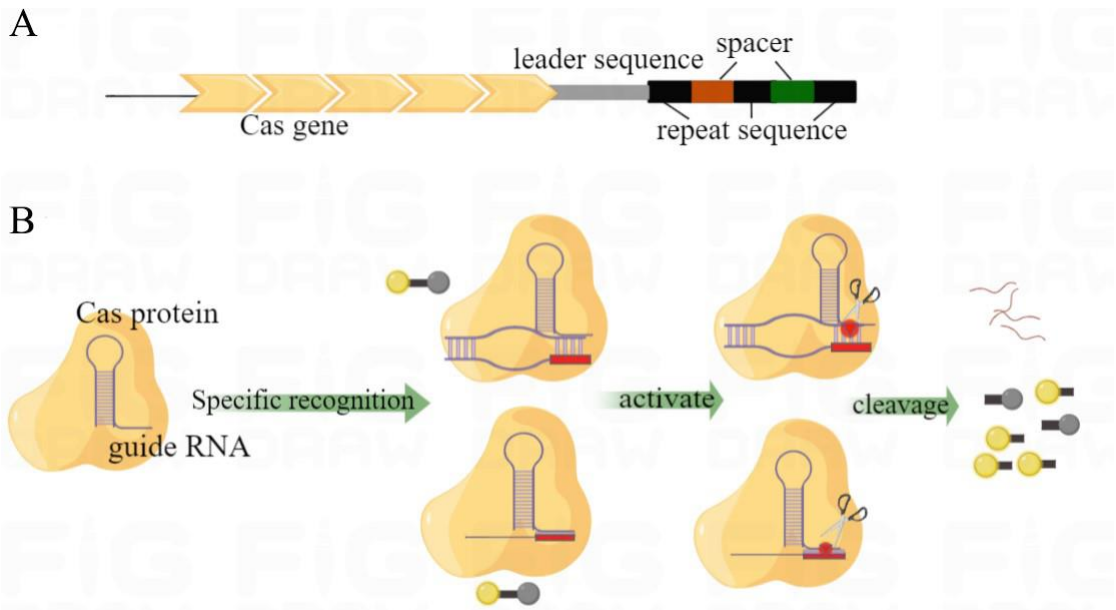


Figure 1: (A) General principle of CRISPR/Cas technique. (B) CRISPR/Cas cleavage principle.

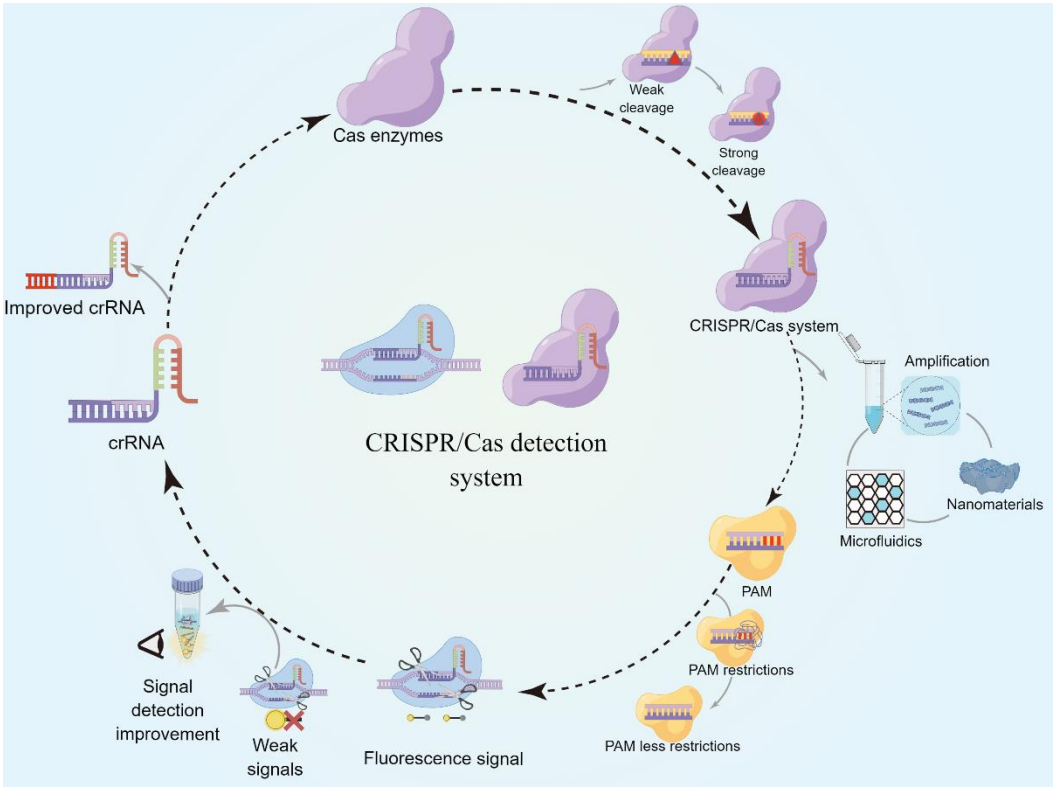


Figure 2 Difference in different CRISPR/Cas systems commonly used in detection.

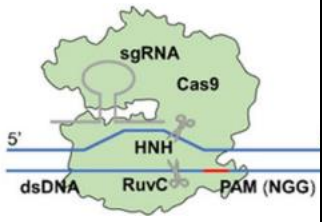
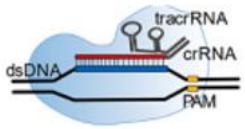
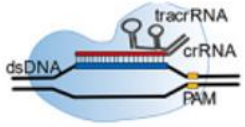
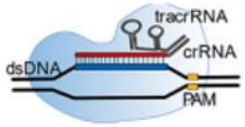
2.2 The classification of CRISPR/Cas system

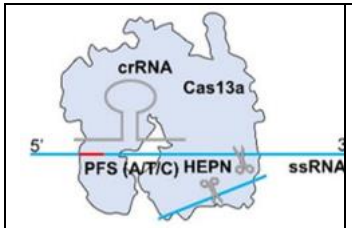
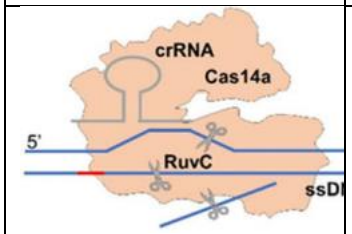
CRISPR/Cas systems can be divided into two classes according to the number of

effector proteins. the CRISPR/Cas system is further split into 6 types and 33 subtypes[5]. Class I includes Type I, III, and IV with multi-subunit effector complex. In terms of the Class II, it contains type II, type V and type VI. Class II systems require only a single effector protein. In addition, Class II has an easier and more organized CRISPR/Cas locus compared with Class I, which makes it easier to reconstitute[5].

Thus, effector proteins of Class II are most extensive used in the field of detection and gene editing. We recapitulate the characteristics and restrictions of the CRISPR/Cas system which are commonly used in detection in table 1. Therefore, existing restrictions and deficiencies in CRISPR/Cas detection systems, we overview improved measures and principles.

Table 1 Features and limits of commonly used CRISPR/Cas system in detection

Cas effector	Type	Endonuclease domains	Guide RNA	Cleavage end	PAM	Target type	Collateral cleavage	Limits
 Cas9	II	RuvC, HNH	sgRNA	Blunt	NGG	dsDNA	No	Sequence constraints，Without collateral cleavage activity，off-target
 dCas9	II	No	sgRNA	No	NGG	dsDNA	No	Sequence constraints，Without collateral cleavage activity，
 Cas12a	V	RuvC	crRNA	Staggered	(T)TTN	dsDNA，ssDNA	ssDNA	Sequence constraints，
 Cas12b	V	RuvC	sgRNA	Staggered	TTN	dsDNA，ssDNA	ssDNA	Sequence constraints

Cas13a		VI	2*HEPN	crRNA	Near U or A	PFS（non-G）	ssRNA	ssRNA	Sequence constraints
Cas14a		V	RuvC	crRNA	Staggered	No	ssDNA	ssDNA	Low detection specificity

* N is any base (A, T, G, or C)

3. Structural parts improvement of CRISPR-Cas systems

3.1 crRNA

During the expression and maturation stages, CRISPR arrays are transcribed into precursor RNA transcripts, which are further processed into smaller RNA units called CRISPR RNAs (crRNAs)[6]. Each crRNAs contains a spacer region flanked by a portion of repeat sequences. These RNAs connect with one or more Cas proteins to come into being an active Cas-crRNA complex[7]. Hence, it is crucial and vital to enhance cleavage power of crRNA. Theoretically, if each of Cas13a nuclease-inactive ribonucleoprotein complex (RNP) is directed to a different region of the same viral target RNA. A single target RNA can activate multiple Cas13a RNPs, which can double the concentration of active enzymes effectively. Fozouni et al. combine two crRNAs with target viral RNA, As the number of activating enzymes increased, the sensitivity and specificity were improved (Figure 2A)[8]. It is reported that extending the 5'end of crRNA can increase the cis-cleavage efficiency of Cpf1 RNP. The main reasons are that crRNA extension enhances Cpf1 delivery through cationic polymers and cationic lipids[9]. It occurs to Nguyen et al and they discovery trans-cleavage activity of LBCas12A remarkably is enhanced by prolonging the 3'- or 5' end of crRNA with different lengths of ssDNA, ssRNA, and phosphorothioate ssDNA without target pre-amplification (Figure 2B)[10]. Similarly, an assay demonstrates that Cas12a trans-cleavage activity can be enhanced by extending a random sequence from the 3'-end of ssDNA (Figure 2C)[11]. The principle is contributed to the enhanced interaction between crRNA and ssDNA activator on account of the dangling end effect[12,13]. Another

strategy to improve guide RNA is to add universal bases aiming to target polymorphic nucleotide sequences (Figure 2D)[14]. Generally, recognition of degenerate mRNA codons by tRNA anticodon loops is implemented by the inclusion of riboinosine (I) nts[15], so inosine as universal bases is applied to gRNA to detect evolutionary HIV-1 DNA target sequences.

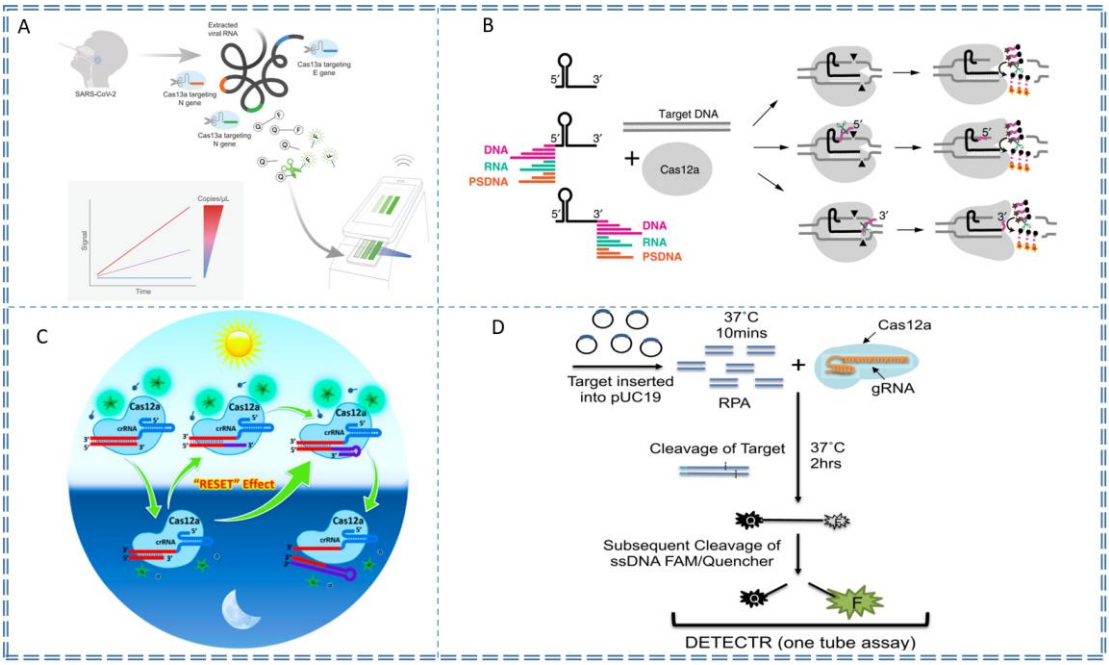


Figure 2 Schematic diagrams for crRNA improvement in CRISPR/Cas detection system. (A) Two crRNAs for SARS-CoV-2 RNA detection. (B) crRNA is extended with ssDNA, ssRNA, or phosphorothioate ssDNA for targeting GFP. (C) Activated CRISPR/Cas12a degrade reporter when ssDNA is extended. (D) Inosine bases into crRNA probes for the Cas12a-based system to detect HIV-1 DNA target sequences.

3.2 Cas proteins

Cas, short for CRISPR-associated protein, can be used to recognize target DNA and obtain a new spacer sequence[16]. The modification of Cas protein can improve the cleavage efficiency and reduce off-target phenomenon. Therefore, an efficient

miniature Cas System (CasMINI) has built from V-F Cas12f (Cas14) syetem, which is compact and smaller than Cas9 and Cas12a (Figure 3A)[17]. Specially, a panel of fusion variants was generated from Cas12f, from which the GFP-activating dCas12f variants fused to the viral protein regulator (VPR) were screened. After 4 rounds of iterations, mutants with nearly 200-fold increased activity over wild-type Cas12f were screened, which used for robust genome editing. A further small minimal functional CRISPR-Cas system has also been described with a molecular weight half that of Cas9 and Cas12a proteins harnessing a single active site for genome editing (Figure 3B)[18]. Likewise, a structure of Cas7-11 in complex with a crRNA and its target RNA is reported using Cryo-EM. Based on structural finding that the additional large insertion (INS) domain keeps away from the target RNA cleavage sites, a series of compact Cas7-11 variants, lacking the INS domain, is engineered to enable transcript knockdown via a single adeno-associated virus (AAV) vector (Figure 3C)[19]. Another improvement approach of Cas proteins is addition of L-proline that can raise Cas12a/Cas13a reaction efficiency and specificity (Figure 3D)[20]. Studies have shown that chemicals such as polymers, amino acids and so on have been used as solvent additives to maintain the stability and solubility of proteins., which in turn influence reaction chemical kinetics[21]. DMSO, glycerol and betaine have a significantly effective enhance in PCR or isothermal amplification reactions[22,23].

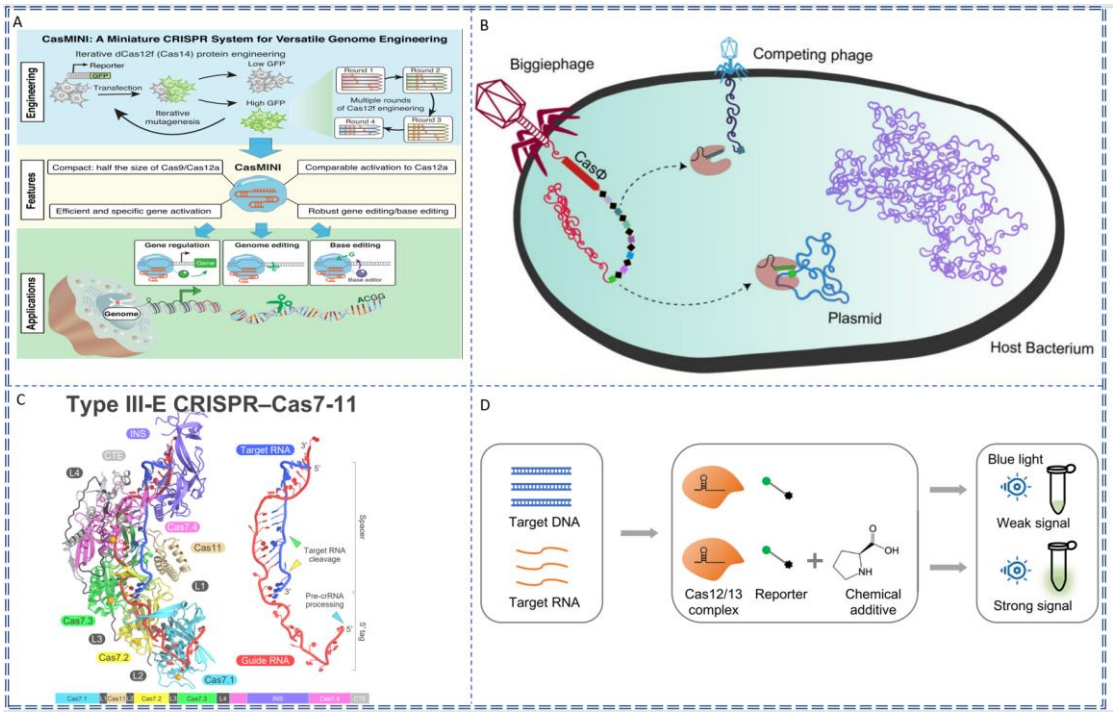


Figure 3 Schematic diagrams for Cas enzyme improvement in CRISPR/Cas detection system. (A) An miniature Cas System for gene editing. (B) Schematic of the hypothesized function of Biggiephage-encoded CasΦ in an instance of superinfection of its host. (C) Structure of the type III-E CRISPR/Cas7-11. (D) The workflow to evaluate chemical additive effect on CRISPR detection system.

3.3 PAM

The Cas protein complex is usually cleaved by binding to the target DNA after recognizing a unique short protospacer-adjacent motif (PAM). CRISPR-targeted recognition requires PAM or protospacer flanking sequence (PFS), which results in sequence restriction and thus reduces the range of detectable sequences[24]. The optimization of PAM in CRISPR/Cas recognition can increase the detection flexibility, broaden the detection target and have a wider range of applications.

Canonical PAMs-mediated cis-cleavage will bring about excess substrate

consumption at low concentrations of substrate. Amplicon accumulation is slow and unstable, leading to delayed or lack of collateral activity. Lu's group designed suboptimal PAM sequences instead of canonical PAMs (Figure 4A)[25]. Suboptimal PAMs exhibited faster sensitivity due to the accumulation of more substrates at the early stage of the isothermal reaction in the one-pot test. Furthermore, an efficiency-enhancing variant (iSpyMac) was designed by means of grafting the PAM interaction domain of SmacCas9 into its mature homolog from *Streptococcus pyogenes* (SpyCas9) in a bioinformatics way, realizing powerful genome editing activities[26]. All adenine dinucleotide PAM sequences can be targeted. Besides, in order to address the problem of broad screening of randomized DNA libraries, Rybnicky's group launched an R software package-Spacer2PAM (Figure 4B)[27]. Spacer2PAM calculates the alterations of phage CRISPR spacers, and the targeted PAM library is obtained after batch alignment and filtering.

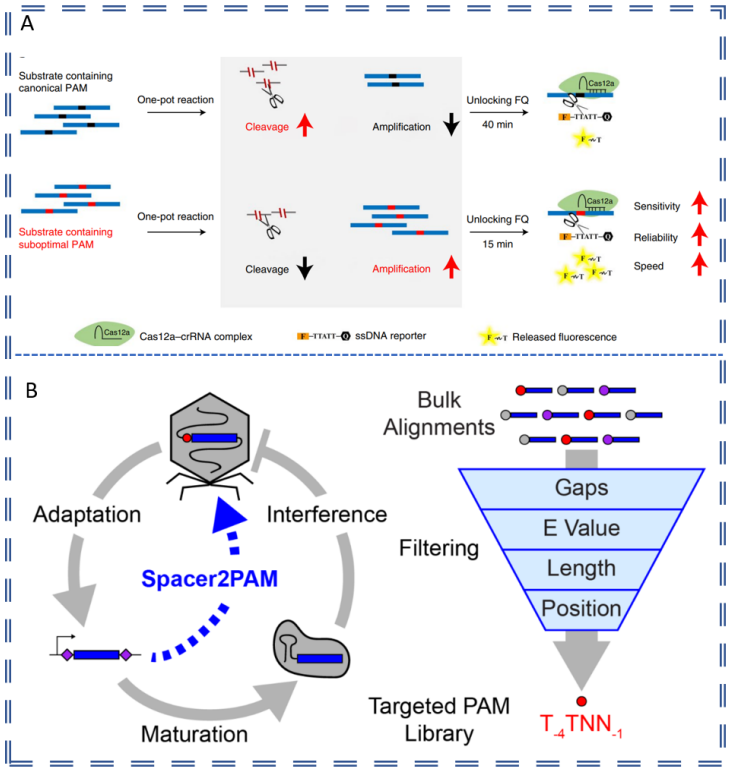


Figure 4 Schematic diagrams for PAM improvement in CRISPR/Cas detection system.(A) Workflow of amplification and cleavage for canonical PAM and suboptimal PAM in one-pot reactions. (B) Calculation workflow of Spacer2PAM.

3.4 Probe

For the probe, its scope has been broadened to targets that were not previously accessible (such as cell phenotypes, cell differentiation stages and diagnosis imaging tools)[28]. The conventional target-oriented fluorescent probes begin with the selection of a target analyte, following fluorophore and quencher attached to the end of the probe that transduce the biological or chemical events into a fluorescent signal by different mechanisms[29]. Whereas, several probes are improved as hairpin structures so as to bind with targets as templates. For example, an allosteric probe (AP) composed of domains of aptamer, primer binding site, T7 promoter. The structure of AP undergoes a conformational change and aptamer domain bind with target in the presence of bacteria. Further, primer forms a band with the target as well, AP is complementary to the target and yield a dsDNA. Subsequently, many ssRNAs are generated by transcription and amplification on account of T7 promoter. At last, Cas13a/crRNA bind with ssRNA and is actived to cleave reporter probe releasing fluorescence signals (Figure 5A)[30]. A hairpin probe of similar structure is described for detecting mutations in serum samples. In detail, this hairpin probe is designed compring a overhang and a single-stranded Nb.BbvCI nicking site. After CRISPR RNP complex matched target. The hairpin structure will change and complement the non-target strand and act as a template to trigger strand displacement amplification (SDA) reaction, which is amplified to

produce a fluorescent signal (Figure 5B)[31]. Apart from the form of hairpin probe, Li et al illustrate another conformational probe (Figure 5C)[32]. The kind of probe consist of two hybridization probes and will not hybridize to each other in a low temperature. If the target exists, they will form a three-way junction and extend into a dsDNA in the presence of a polymerase. Next, activity of Cas12a is activated to yield amplified fluorescence signal. Moreover, another type of probe is designed compring two probes as well (Figure 5D)[33]. Under circumstance of without ligation, DNA elongates from a forward probe to a phosphorothioated-hairpin probe, after which sequential DNA elongation occurs at the nicking site of the PS probe and in the self-primer region, binding to CRISPR/12a yields cleavage activity.

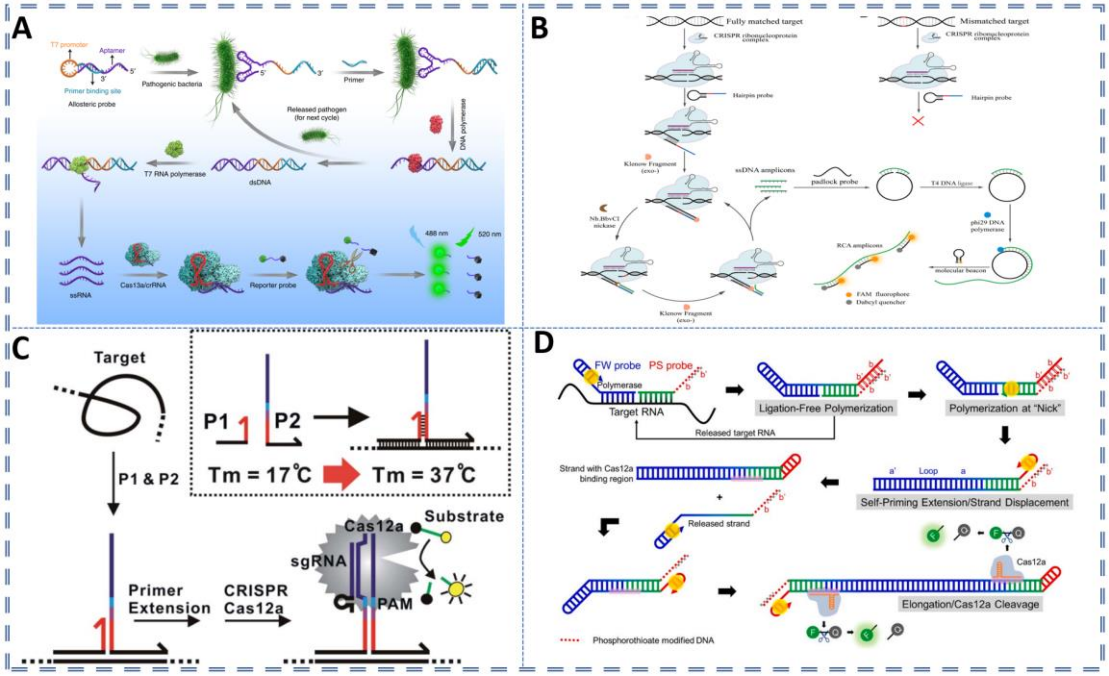


Figure 5 Schematic diagrams for probes improvement in CRISPR/Cas detection system. (A) Workflow of AP for pathogenic bacteria. (B) Working principle of hairpin probe for binding to noncomplementary strand and triggering SDA reaction.(C) Working principle of P1 and P2 probe used for detection of nucleic acids.(D) Working principle

of FW and PS probe used for nucleic acid amplification.

3.5 Activator

Activators are designed to activate the CRISPR/Cas system for target cleavage. The basic principle is that the activator itself or part of the activator binds and complements crRNA to unlock CRISPR/Cas cleavage activity, posing signal amplification. Some DNA-based activators are designed for the detection of protein/small molecules[34,35]. For instance, Kim et al harness small molecule-modified activator DNA to complement crRNA freely when the proteins are absent, starting the collateral cleavage activity of CRISPR/Cas12a (Figure 6A)[36]. In addition, terminal deoxynucleotidyl transferase (TdT) and small molecule-SAM can also be constructed to induce the emergence of activators to trigger CRISPR/Cas detection system(Figure 6B,C)[37-39] .

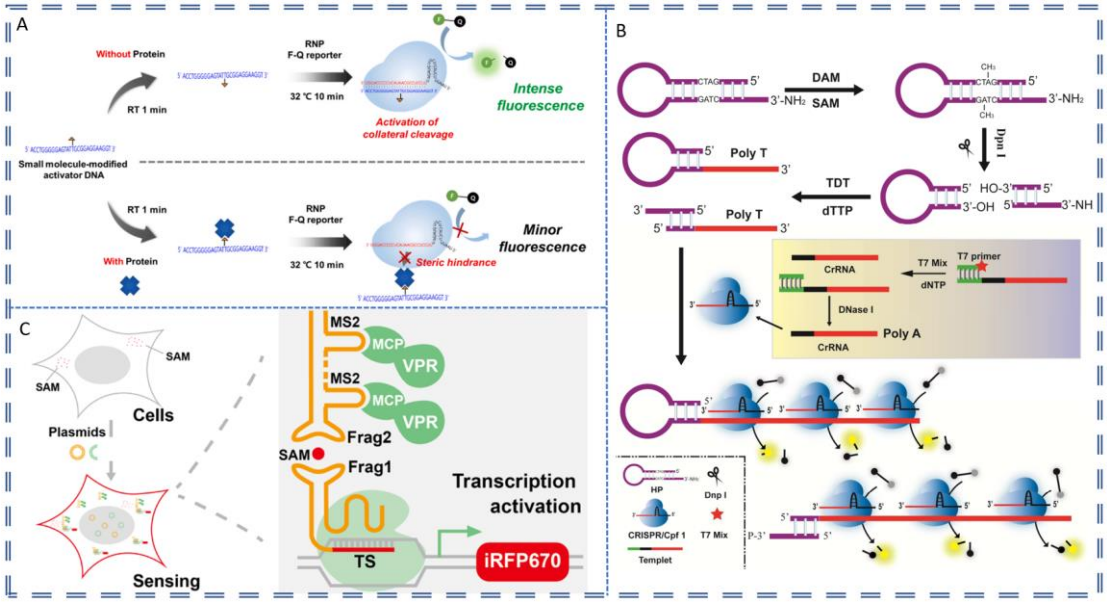


Figure 6 Schematic diagrams for activators improvement in CRISPR/Cas detection system. (A) Schematic illustration of small molecule-modified activator DNA for protein/small molecule based on the CRISPR/Cas12a. (B) Schematic diagram of TdT-mediated based on the CRISPR/Cpf1 for Dam MTase activity detection. (C) Schematic

of SAM - mediated CRISPR-dCas9 transcription system for detection of small molecules.

4. CRISPR/Cas systems couple with other technologies

Improvements to the CRISPR/Cas system itself have improved detection sensitivity, accuracy and efficiency. With the rapid progress in nucleic acid amplification technology, microfluidic, nanomaterials, etc. These techniques are offering the opportunity to detection nucleic acid with precision and creating new opportunities for further refinement of CRISPR/Cas-based detection system, indicating the kind of detection technology does not rely on sophisticated equipment, which makes CRISPR/Cas detection system is faster and more sensitive.

4.1 Nucleic acid amplification techniques

4.1.1 PCR

Nucleic acid amplification technologies have been widely applied to detect a variety of nucleic acids coupling to CRISPR/Cas system because of remarkable advantages, including quick response time as well as high sensitivity (Table 2). PCR technology the most commonly utilized technique in molecular diagnostics for detecting small amounts of target DNA or RNA fragments[40]. The combination of PCR and CRISPR can further promote the detection sensitivity and specificity. Generally speaking, it mainly uses PCR to amplify specific gene fragments, and then uses CRISPR/Cas to identify the amplified gene, cut the reporter and emit fluorescence. To sensitively detect samples, a quantitative polymerase chain reaction(qPCR)-based method is developed to integrat into the CRISPR/Cas system assisting amplification and recognition, which

is operable and effective in practical detection (Figure 7A)[41,42]. Furthermore, quantitative reverse transcription polymerase chain reaction (RT-PCR) is also involved in the detection of CRISPR/Cas system. Recently, epidemic viruses are detection through RT-PCR-mediated amplification and CRISPR/Cas-based visualization (Figure 7B)[43,44]. RT-PCR magnifies target regions of SARS-CoV-2 genome coupling to CRISPR/Cas technology to verify with the improvement of sensitivity and portability. However, there is a technical challenges lie ahead in the fast detection. To this end, an ultrafast RT-PCR and CRISPR method for instant viruses detection is proposed (Figure 7C)[45]. Both RT-PCR and CRISPR system are in one tube. After ultrafast RT-PCR deal with RNA within 5 min, CRISPR/Cas12a system is activated to cleave.

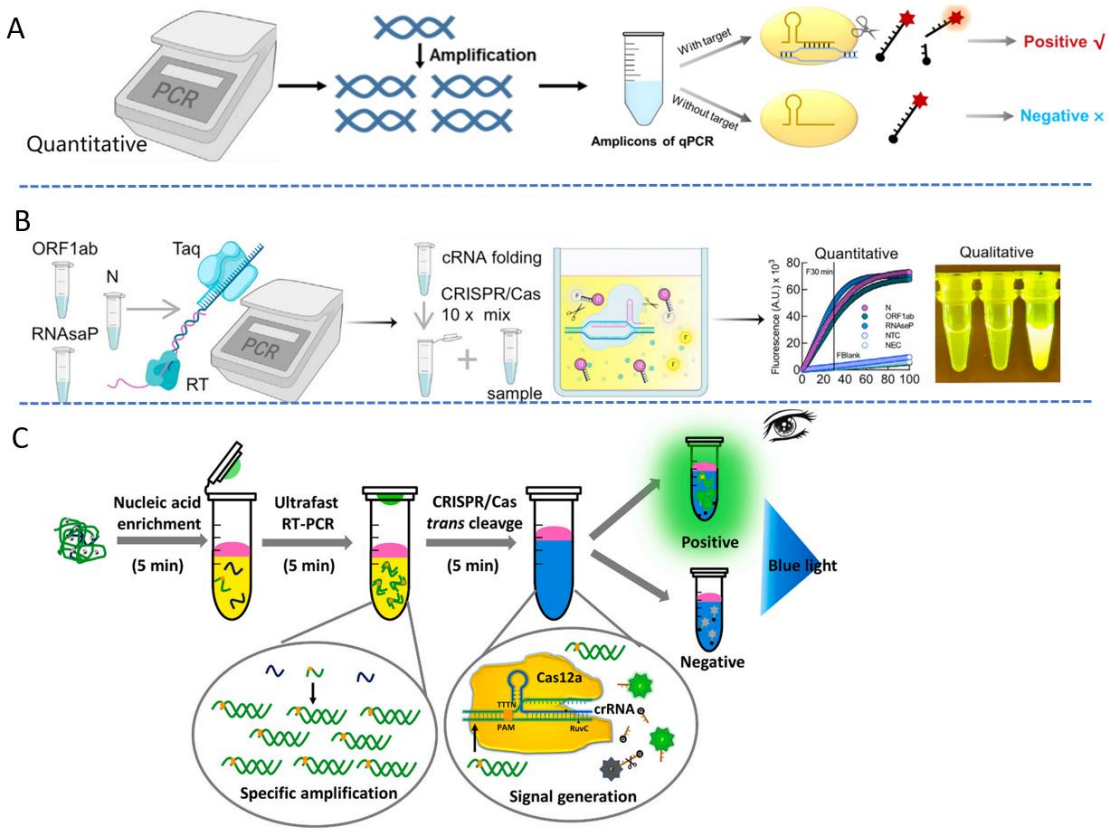


Figure 7 Schematic diagrams for PCR combined CRISPR/Cas system with improving detection efficiency.(A) Workflow of qPCR-based samples detection. (B) Workflow of

RT-PCR-based RNA viruses detection. (C) Workflow of ultrafast RT-PCR-based samples detection.

4.1.2 LAMP

PCR-based amplification and detection methods are highly sensitive and specific, but require expensive equipment and skilled performance, which limits their comprehensive applications[46].Compared with PCR, isothermal amplification of nucleic acids has emerged as a promising method for rapid and portable detection, such as loop-mediated isothermal amplification (LAMP), strand displacement amplification (SDA), recombinase polymerase amplification (RPA), etc. The required equipment is relatively simple, which can achieve rapid on-site detection. These emerging tools can be as substitutes for PCR to apply in the diagnostic field associating with CRISPR/Cas system.

For RT-LAMP-based CRISPR detection, both primer-specific amplification and guide RNA were utilized to fulfill directional detection. RT-LAMP was employed for gene amplification together with CRISPR/Cas for specific recognition. Only in the presence of specific crRNA and RT-LAMP amplicon can the reporter be cleaved. LAMP is integrated with CRISPR/Cas-based assays into a single tube and demands only a single fluid processing step[47]. Now, LAMP is increasingly combining with CRISPR/Cas to detect analytes[48-53]. For example, an AuNP-based visual assay that combines with Cas12a-assisted RT-LAMP amplification is developed by Zhang et al, which is rapid and on-site diagnostic assays for high volume screening[54]. In this protocol, RT-LAMP reverse transcribes and amplifys specific viral gene acquiring

amplicon, thereby be recognizing by Cas12a/gRNA. Another one-pot form is as similar as former which is established (Figure 8A)[55]. LAMP amplification reaction is implemented using the three primers sets, containing a PAM site that is recognized by the corresponding Cas12b/sgRNA system to cleave. It sheds light on Zhu et al. Zhu's team perform a approximate job in the light of previous research. Target DNA is enriched by LAMP technique adding a PAM site inside to make LAMP products recognized[56]. In addition, LAMP amplification can be employed to detect samples which is from a polypropylene (PP) bag that harbors lysis chamber, cleaning chamber and amplification chamber to improv portability (Figure 8B)[57]. Besides these forms, LAMP, CRISPR/Cas, one-pot format are also connected with digital platform[58]. Ding's team develop RT-DAMP instead of RT-LAMP, All the reagents are prepaerd in a tube, After mixed, the mixture is dispersed into digital platform. The fluorescence of the positive can be read out from microwells (Figure 8C)[59].

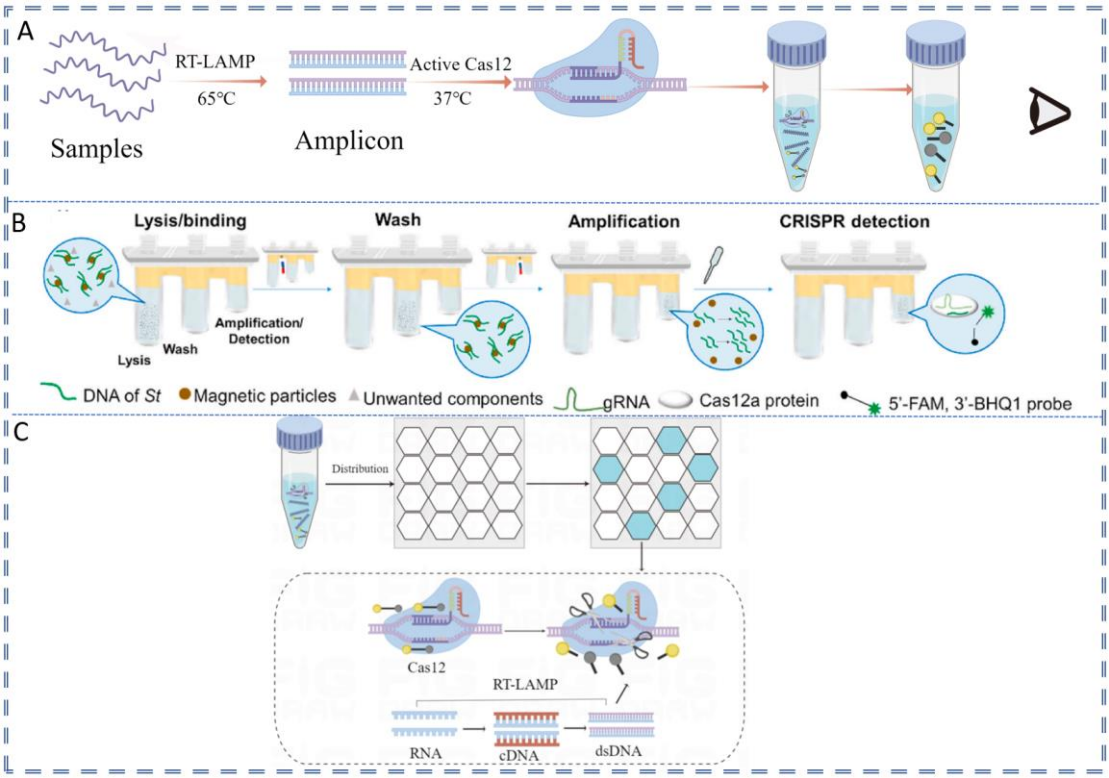


Figure 8 Schematic diagrams of LAMP combined with CRISPR/Cas system detection. (A) Workflow of LAMP-based samples detection. (B) Workflow of nucleic acid detection in the PP bag using LAMP and CRISPR/Cas. (C) Workflow of LAMP-based samples detection coupling to digital platform.

4.1.3 RPA

RPA can amplify the template at about 37℃ depending on the strand displacement activity of recombinase. RT-RPA has been coupled with CRISPR for the detection of SARS-CoV-2[60,61]. The reaction temperature of RT-RPA is close to that of Cas12. Hence, RT-RPA isothermal amplification and CRISPR/Cas12 detection are regarded compuponible in principle. RPA is the same as LAMP that can be incorporated into one-pot making it possible for RPA to address the false-positive problem owing to amplicon contamination ((Figure 9A)[62-66]. Based on conventional one-pot detection method, Lin et al demonstrate that the addition of glycerol remarkably boosts the detection

efficiency of one-pot RPA-CRISPR/Cas12a with 100-fold enhancement[67]. For one-pot RPA-CRISPR/Cas12 improvement, Hu's team pursues to clear a key hurdle in a photocontrolled form that the CRISPR-Cas12a system will be prevented due to the complementation between crRNA and photocleaved linker. CRISPR-Cas12a detection system can be flashily activated by illumination after finishing the RPA reaction. They aim to fulfill for purpose of improvement in sensitivity[68]. To solve the problem of quantification, After the reactant is prepared in one pot, one-pot mixture is generally distributed into digital platform, then, DNA is amplified by RPA In the temperature of 37°C, thereby triggering Cas12 endonuclease that is complementary to exposed target sites (Figure 9B)[69,70].

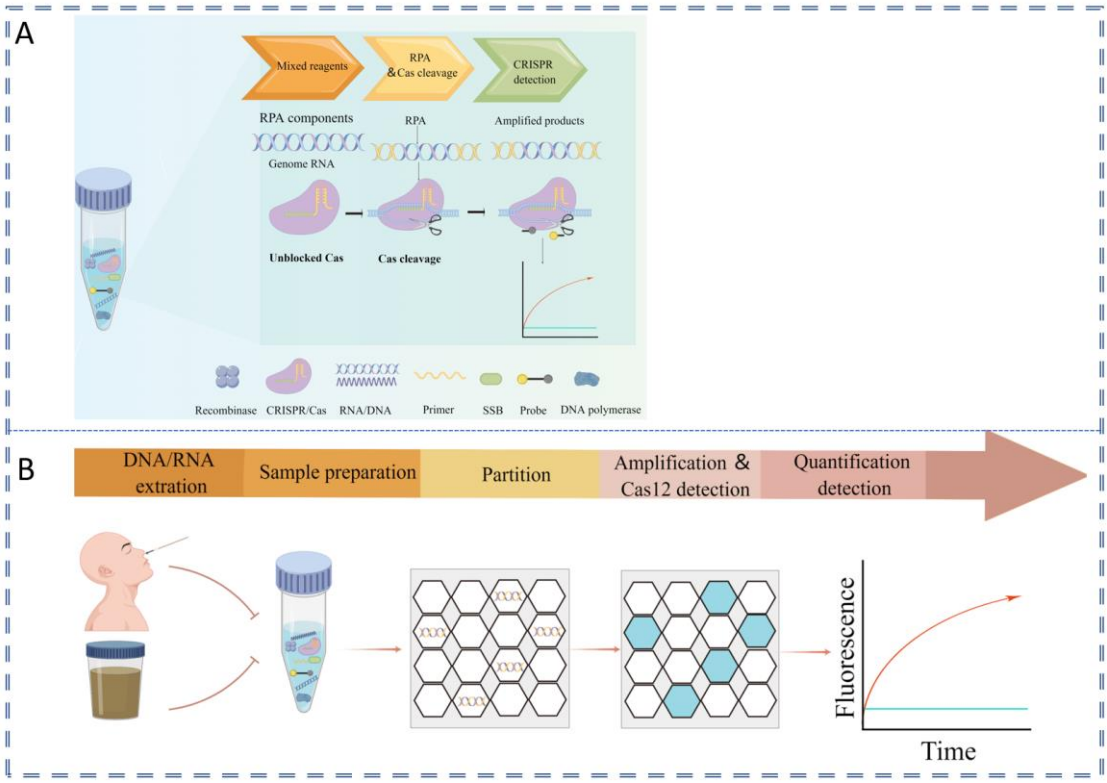


Figure 9 Schematic diagrams of RAP combined with CRISPR/Cas system detection. (A) Workflow of one-pot detection form based on RPA and CRISPR/Cas. (B) Workflow of digital detection form based on RPA and CRISPR/Cas.

4.1.4 SDA

SDA is an enzymatic reaction-based isothermal amplification technology in vitro. A dsDNA contains the target for detection and nicking enzyme for recognization nicking. With the assistance of various nicking endonuclease cleaves one of the strands in dsDNA, then, a new double-strand is created through a polymerase-induced strand displacement process. If the target is present, it will initiate the second cycle, producing amounts of amplification. Concurrently, the activation sequence of the CRISPR/Cas is released, the complementary sites of target activate the trans-cleavage activity of CRISPR-Cas to make ssDNA reporter cleaved and unleash the fluorescence signal (Figure 10)[71,72].

Based the detection of SDA coupling with CRISPR/Cas system, it is more and more used to applying to a varity of fields[73-75]. For instance, Zhou et al harness SDA to CRISPR/Cas12a for detection of DNA methylation with higher sensitivity[76]. Namely, methylation levels as low as 0.1% are detectable in excess unmethylated DNA. Another example of SDA coupling with CRISPR/Cas is SDA amplifies miRNAs directly rather than reverse transcription in the presence of DNA polymerase, which decrease the hazard of non-specific amplification, providing a sequential amplification that improves the sensitivity for miRNA detection[77]. Wang's team has a counterpart in miRNA detection[78]. Also, a CRISPR/Cas-based biosensor is exploited associated with quadratic SDA, which makes the assay with the characteristics of rapidity and isothermal[79].

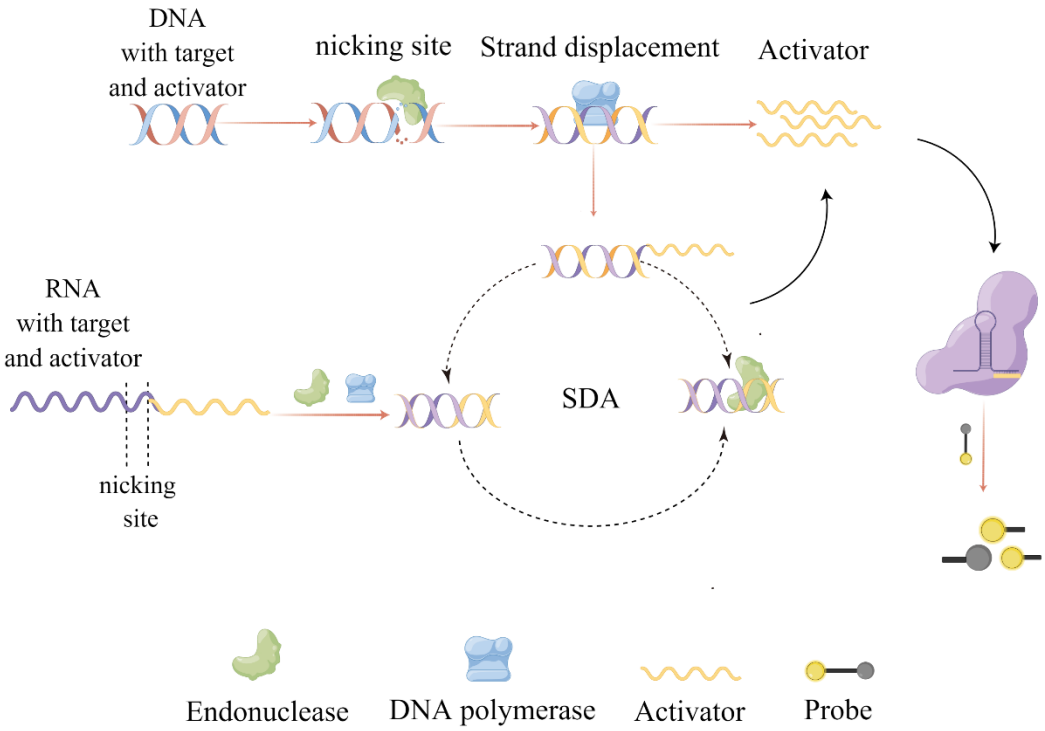


Figure 10 workflow of SDA combined with CRISPR/Cas system detection.

Table 2. Representative nucleic acid amplification techniques combine with CRISPR/Cas system for nucleic acid detection

Acid amplification techniques	Samples	Detection performance	Time and temperature	Detection rate
PCR	ASFV; rice; SARS-CoV-2 mutations	sensitivity>95%	<2h; 95° C-37° C	100%
LAMP	Bacteria; viral RNA; Meat Products	LOD:< 5 copies/μL (viral RNA); LOD:10 pg pure genomic DNA	<70min; <70° C	100%
RPA	virus	Linear range :0.5-2027 copies/μL	<60min; 37° C	100%
SDA	DNA methylation; microRNAs; DNA Glycosylase	LOD:1.28 × 10-13 M（DNA methylation）;4.24 × 10^{-9} UμL^{-1}(DNA Glycosylase)	<60min; 37° C	100%

4.2 Microfluidic technology

Microfluidic chip is a miniaturized integration technology that integrates the basic

operation units of sample preparation, reaction, separation and detection for life science application analysis into a single chip. A small number of sample volumes can be added to realize the parallel automatic analysis of several samples in a short time. Microfluidic chips has the characterestic of speediness, precision, versatile integration and high-throughput, which has obtained a lot of research in the detection of imported infectious diseases. With the continuous development of microfluidic chip technology, a variety of microfluidic chips have been emerged, such as paper-based microfluidic chip[80,81], digital microfluidic chip[82,83], POC microfluidic chip and so on. Microfluidic technology, with its precise manipulation of fluids and high integration, provides unprecedented opportunities for CRISPR/Cas-based assays. The samples were loaded into a microfluidic platform with storage cavities and channels by harnessing different cleavage preferences of multiple Cas proteins or orthologs to detection.

Microfluidic chip combines CRISPR/Cas that has been widely used for high throughput detection[84-86]. For instance, a microfluidic system (MAPnavi) is developed for the rapid diagnosis of respiratory viruses by Liu et al (Figure 11A)[87]. Size of MAPnavi is small enough to accommodate 8 samples and it contains 8 separate units with the characterestic of seale and automation to avoid contaminating. After the samples are collected, the RNA extraction is loaded onto the chip-based MAPnavi, where it is amplified and detected though RT-RPA and CRISPR/Cas system. Another self-contained microfluidic system is also involved in pollution-free detection (Figure 11B)[88]. The microfluidic system is composed of a microfluidic chip containing reaction chamber, collection chamber, detection chamber and a hand warmer instead of

electricity to incubate the microfluidic chip, achieving simple, affordable diagnostics for POC detection. Additionally, microfluidic array format is designed for high-throughput, multiplexed detection[89-91]. A nucleic acid detection platform is developed termed CARMEN[92]. In this platform, the detection mixture combines with different fluorescent color codes served as optical recognizer. After emulsification of each color-coded solution, all samples and droplets of the detection mixture are pooled into a single tube and load into a micropore array chip in a pipetting step for identification by fluorescence microscopy. But it is different to use be because of the labor-intensive protocols. To meet the more public health requirements, Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids (mCARMEN) emerge to eliminate the needs of color-coding and dropletization and the samples and detection assays are shifted into chip reaction chamber viaindividual channels on the IFC. After mixed, fluorescence signals are measured on the Fluidigm Biomark (Figure 11C)[93]. Also, CRISPR/Cas-based microfluidic paper is involved in detection (Figure 11D)[94]. In this assay, paper-based microfluidic analytical device（μPAD）is divided into two layers. The substratum is back pad with 0.2mm (thickness). The superstratum is hydrophilic filter paper with 0.2mm (thickness) and has hydrophobic wax on the surface. The substratum contains a loading and reaction zone for the addition of surface-enhanced raman scattering (SERS) probe and CRISPR/Cas and detection zone for analysis of results. Furthermore, droplet digital-microfluidic technology combines with CRISPR/Cas as an emerging start-up not only providing a powerful platform, but a large-scale biological analysis. Cui et al design a microfluidic device, including reaction

mixing zone for discretizing mixture and injection zone for the injection of MgOAc to unlock the digital reaction via an external pressure source to undertake a digital output (Figure 11E)[95]. Yu's team did a near work through a SlipChip design that involves movable loading position and partitioning position, followed by adding and moving reagents twice into partitioning position to pursue a digital analysis (Figure 11E)[96].

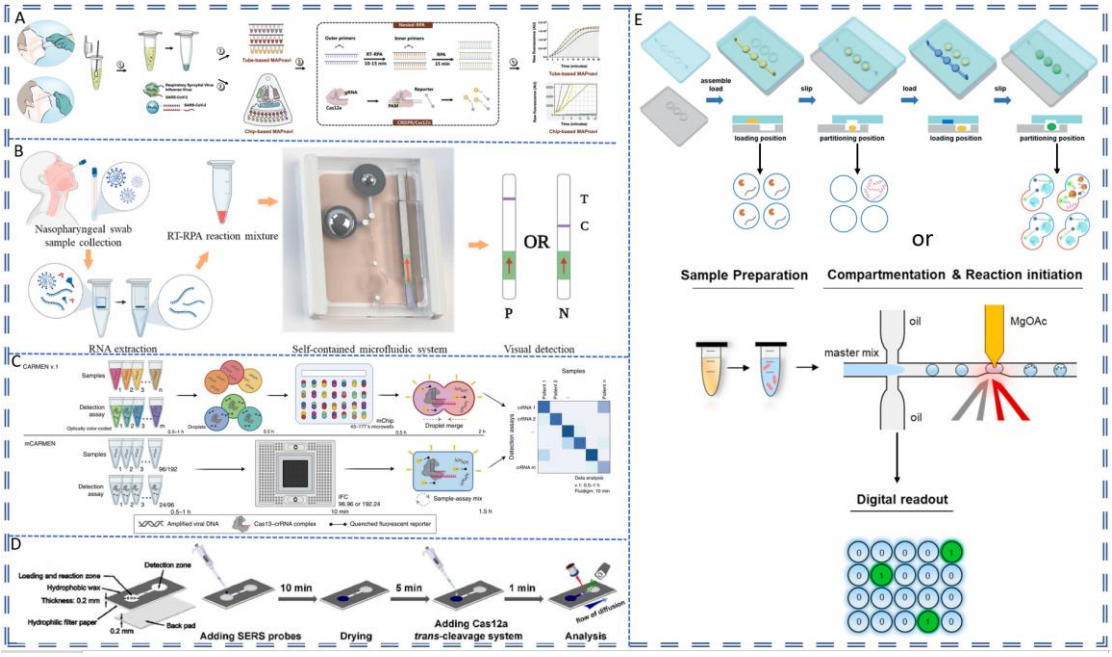


Figure 11 Schematic diagrams for microfluidics coupling with CRISPR/Cas system (A) Workflow of the MAPnavi-based on nested RPA and CRISPR/Cas12a. (B) Workflow of the self-contained microfluidic and CRISPR/Cas system for virus detection. (C), Schematic of CARMEN v.1 (top) and mCARMEN (bottom) coupling with CRISPR/Cas system. (D) Workflow of the μPAD-based and CRISPR/Cas12a. (E) Workflow of digital-microfluidic technology and CRISPR/Cas for detection.

4.3 Nanomaterials

With the progress of nanotechnology, nanomaterials have been gradually used for detection due to their excellent physical and chemical properties, such as peculiar

optical properties, specific catalysis, and strong chemical reactivity. Through the sensitive optical properties of the signal output, combined with the CRISPR/Cas system, the target signal is enhanced. Upconverion nanoparticle (UCNP) as a subclass of lanthanide-doped luminescent nanocrystals is created with a polyacrylic acid (PAA) coated and a reporter conjugated to be cleaved owing to the activation of Cas12 (Figure 12A)[97]. And the build of luminescent resonance energy transfer (LRET) avoids false signals due to the generation multiple optical information. PAA-SUCNP is designed in the form of sandwich structure consisting with NaYF4 that is the substrate with the highest fluorescence efficiency and PAA polymers that is the characteristic of hydrophilia and dispersity. Au nanoparticles (AUNPs) have the feature of local surface plasmon resonance, namely, when the free electrons on the surface of AuNPs are stimulated by the irradiation light, the surface plasmon resonance effect will be generated, a characteristic resonance peak will be displayed in the UV-visible region. Location and shape of the characteristics of the resonance peak will change as the particle spacing, particle size. If the distance between gold nanoparticles is too short，AuNPs manifest plasmonic effects similar to those of aggregates. The appearance colors present changes from red to purple. Base on the property, to optimize the performance of the CRISPR/Cas-based biosensors, 'hot spots' are generated at nanoscale junctions between two compactly coupled AuNPs to exhibit signal changes resulting from the cleavage of CRISPR/Cas (Figure 12B)[98,99]. Graphene nanoelectronics offer an alternative platform for the sensing applications based on CRISPR/Cas[100]. Graphene has a large specific surface area and can act as a strong

adsorbent. On the other hand, graphene has a high internal charge carrier mobility, which can boost electrochemical current signal. Based on these properties, graphene combines CRISPR/Cas are employed to enhance signal changes after target nucleic acid is bound with sgRNA/dCas9 followed the interaction between dRNP and target DNA (Figure 12C)[101]. Nanomaterials-based artificial enzymes, which are named nanozymes, have the catalytic properties of enzymes[102]. The reporter with FAM and biotin bond to anti-FAM antibody is intact in the absence of target analyte. Ulteriorly, nanozymes catalyze the oxidation and color development of chromogenic substrates[103].

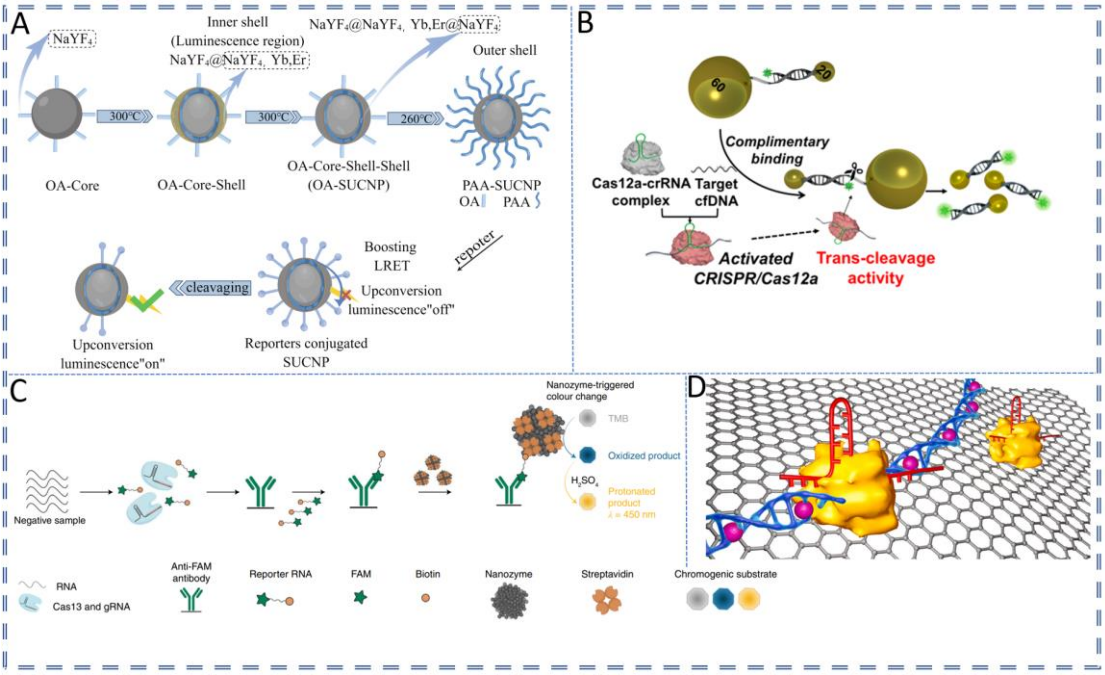


Figure 12 Schematic diagrams for nanomaterials coupling with CRISPR/Cas system (A) Workflow of the PAA-SUCNP-based and CRISPR/Cas12a. (B) Workflow of the AUNPs-based and CRISPR/Cas12a. (C) Graphene-based detection coupling with CRISPR/Cas.

5 Construction of CRISPR/ Cas-based biosensor

Biosensors have the characteristics of strong specificity, high sensitivity and low

determination cost. CRISPR/Cas-based biosensor technology due to efficient targeting binding and programmability is developed as the tool for swift detection. Several common sensors include fluorescence biosensors, colorimetric biosensors, electrochemical biosensors and lateral flow sensors those whose further improve the sensitivity and specificity of CRISPR/Cas detection (Table 3).

Table 3. Several biosensors for improvement of CRISPR/Cas detection

Type	Cas effector	Amplification method	Detection steps	Isothermal	Dynamic Range	LOD	Multiple detection	Quantitative	Assay reaction time	Bulky instrumentation required
Fluorescence biosensors	Cas12a	primer exchange reaction (PER)	One step	Yes	0.005-0.1U/mL	0.00061U/Ml	No	No	<125min	No
		SDA			10pM–500pM	14.4fM	Yes	No		No
		PER			0.001-80ng m^{-1}	0.92pgmL^{-1}				
		SDA			1fM-50nM	1fM.	No	Yes		No
		PCR			0.001-80 ng mL^{-1}	0.92pgmL^{-1}	No	No		No
		No	Three steps	No	0.5100pM	0.54pM	No	No		For PCR
		strand displacement	One step	-	0fM-10pM	4.1fM	No	Yes		No
			One step	Yes						No
Colorimetric biosensors										
Electrochemical biosensors										
Lateral flow sensors										

5.1 Fluorescence biosensors

Fluorescent biosensor indicates that takes advantage of materials with fluorescence capacity to label the target required to be detected and whether the fluorescent signal is displayed that is used as the detection result[104]. Coupling with CRISPR/Cas, it is simple in apparatus and the speed is fast in response. For example, in the presence of the analyte, accompanied by isothermal amplification, the signal is continuously cyclically amplified and the fluorescent signal is restored after trans-cleavage of the activated CRISPR/Cas system, building a facile and sensitive approach[105]. Similar to the above assay, based on SDA, a mass of ssDNA is generated to compelled the trans-cleavage activity of CRISPR/Cas12a. As a result, the reporters of ssDNA are cleaved to yield drastically fluorescent signal, which can improve the detection sensitivity[106]. In the same way, Zhang's team and Pu's team construct analogical fluorescence biosensors for cascade amplification detection (Figure 13A)[107,108]. Another fluorescence sensing strategy is designed that MXenes are added as fluorescence quenchers to facilitate the quenching of ssDNA-FAM and the reduction of the fluorescence background signal (Figure 13B)[109]. Besides, a novel fluorescence biosensor is established by Tao et al based on copper nanoclusters (CuNCs) which is applied as the luminescent nanoprobes for much higher sensitivity and more rapid efficiency if the target is absent (Figure 13C)[110]. In addition, a DNA walker with magnetic nanosphere is proposed to construct fluorescence biosensor for “one-to-many” releasing multiple activators with the assistance of the nicking endonuclease to unlock the trans-cleavage activity of CRISPR/Cas system (Figure 13D)[111].

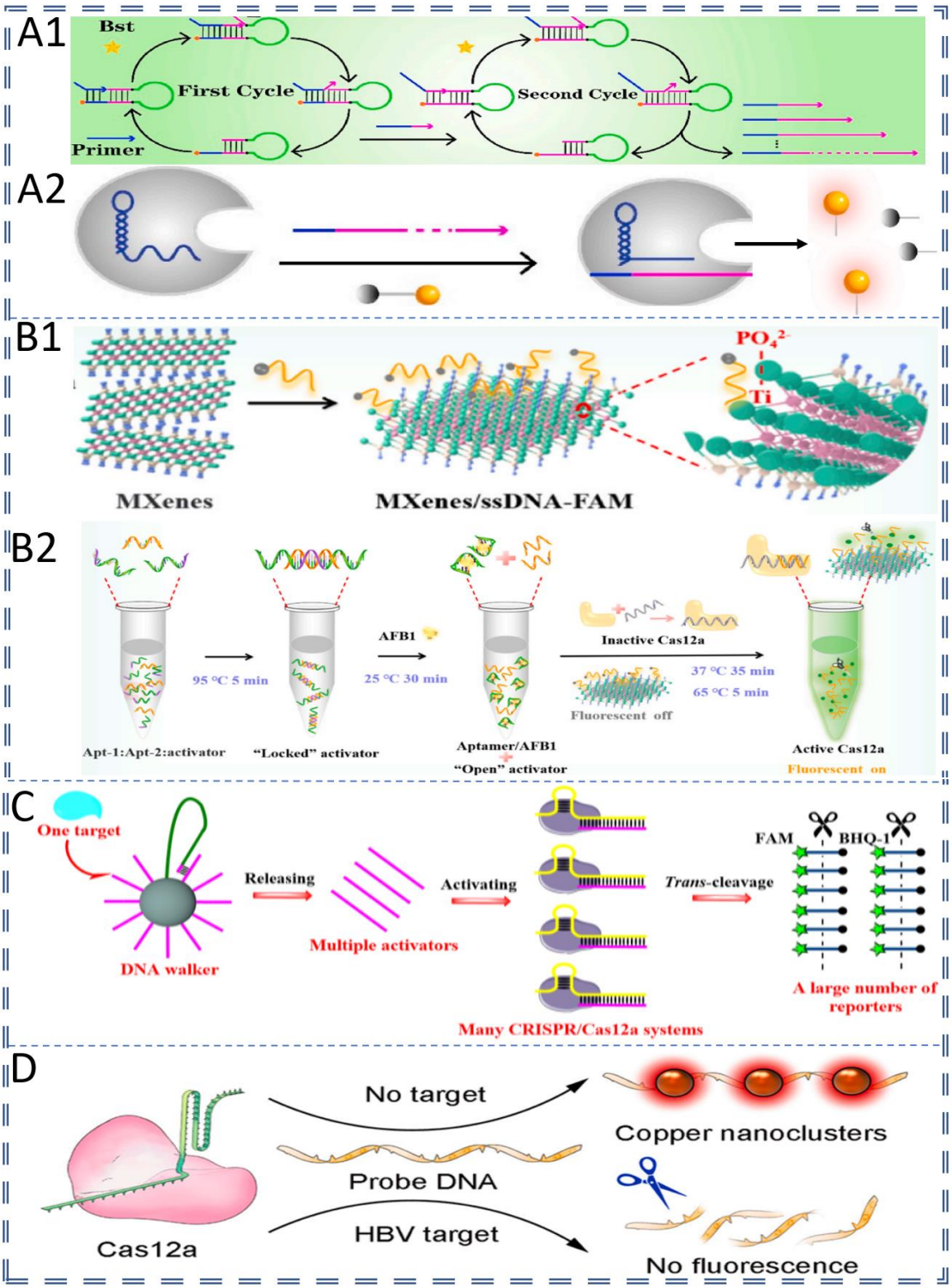


Figure 13 Schematic diagrams for constructed CRISPR/Cas-based fluorescence biosensors. (A) Fluorescent signal amplification strategy based on cascade amplification coupling with CRISPR/Cas. (B) Schematic illustration of the fluorescence biosensor based on CRISPR/Cas12a integrated MXenes for detection. (C) Scheme for the Cas12-CuNCs-based detection. (D) Schematic illustration of the

fluorescence biosensor based on CRISPR/Cas12a using DNA walker for detection.

5.2 Colorimetric biosensors

CRISRP/Cas-based colorimetric biosensors visually detect objects with different colors by using the reflectance difference as the detection principle. Compared with fluorescent biosensor that requires additional tools to read the fluorescence values, colorimetric analysis-based biosensor enables visual detection with the naked eye. Moon et al first added viral lysate and biotin-PAMmer, then added streptavidin-HRP and TMB chromogenic reagents. After streptavidin bound to biotin-PAMmer, resulting in a color change by reason of the oxidation of 3,3′,5,5′-tetramethylbenzidine (Figure 14A)[112]. While this method lack of sensitivity. To overcome deficiencies and shortcomings, recently, many researchers engage nanomaterials integrate colorimetric biosensors into CRISPR/Cas system for signal enlargement of detection results aiming at visual test. For example, in the presence of the analyte, the aptamer can separate from DNA sequence, the released oligo hybridized with the hairpin DNA loop and open the hairpin structure. The primers thereby bound to the DNA hairpin structure, triggering the SDA process. With the help of polymerase, the extended ends of the hairpin DNA form a terpolymer with Cas 12a/crRNA to performed non-specific cleavage of AuNPs-linker ssDNA and generated visual color changes (Figure 14B)[113]. Another improved strategy colorimetric biosensor-based with CRISPR/Cas was to use probe DNA modulation of the catalytic behaviors of Ag/Pt nanoparticles that was modified by Ti3C2Tx Mxene nanostructures to perform the first and second signal amplifications (Figure 14C)[114]. AuNPs−DNA probe was designed used for visual inspection depending on its optical properties when the target was present (Figure 14D). Based on the property of AuNPs−DNA probe, magnetic beads-mediated colorimetric assay offered a quick and easy method for nucleic acid detection, streptavidin-embedded

magnetic beads (SA-MBs) could not enrich AuNP bioprobe after CRISPR/Cas12 was activated following Biotin-P1 was severed, so the supernatant would show red (Figure 14E)[115].

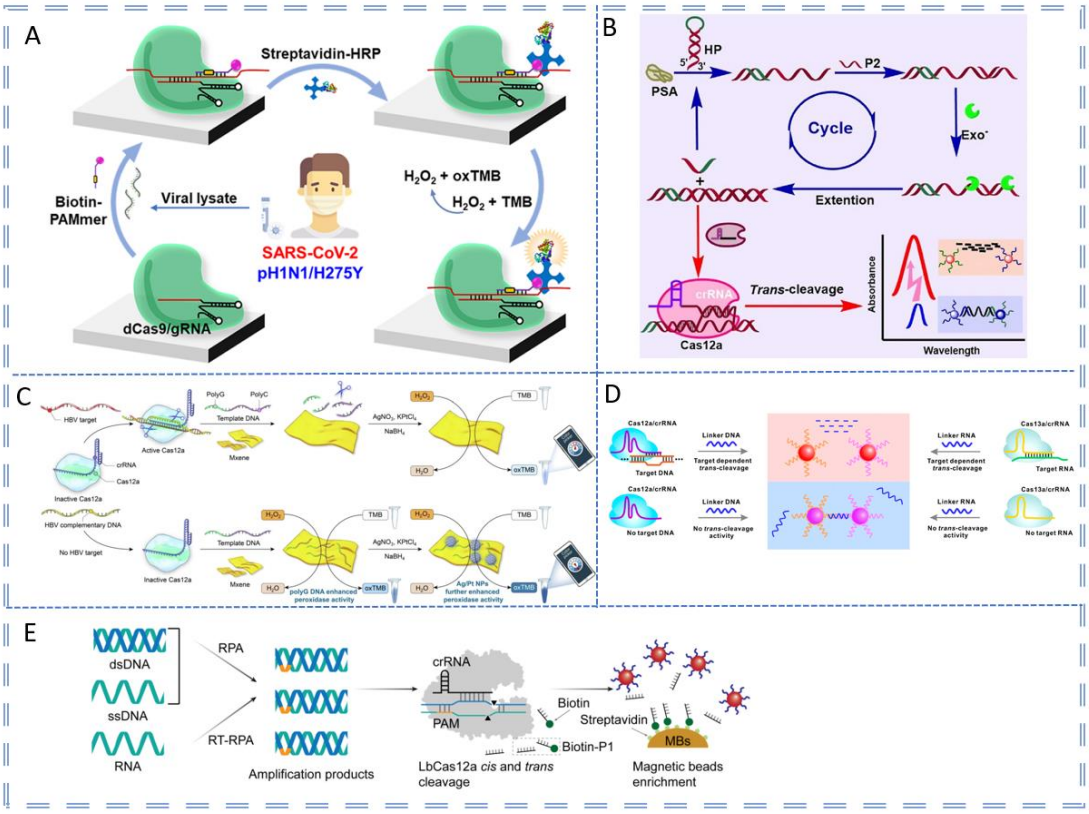


Figure 14 Schematic diagrams for constructed CRISPR/Cas-based colorimetric biosensors. (A), (B), (C), (D) (E)Schematic illustration of the colorimetric detection based on CRISPR/Cas.

5.3 Lateral flow biosensors

Lateral flow strips use DNA or RNA reporter genes with fluorescein and biotin markers at the ends. At one end of the strip was streptavidin, which binds to biotin, and at the other end was an anti-fluorescein antibody labeled with gold nanoparticles, which binds to the reporter gene fluorescein. After the CRISPR complex recognize the target gene, the reporter gene will be cut to release the luciferin and move along the strip to the other end. Sample lines appear on the strip to indicate the detected target molecules. The detection samples can be determined whether the detected samples are positive or

not according to the strips on the strip, and visual detection can be conducted directly. Based on the principle, Gong et al. made improvements. They modified the primers with thiophosphates and made the gold-nanopaticle strip optimized in order that achieved accurate detection effect using CRISPR-Cas12a system (Figure 15A)[116]. Analogically, Cao'steam established lateral flow strip with the characterestics of automation and portable based on CRISPR/Cas13a and microfluidic chip (Figure 15B). Ulteriorly, a composite probe was designed with DNA and antibody binding to CRISPR-Cas12 system to direct identify reporter visually (Figure 15C)[117].

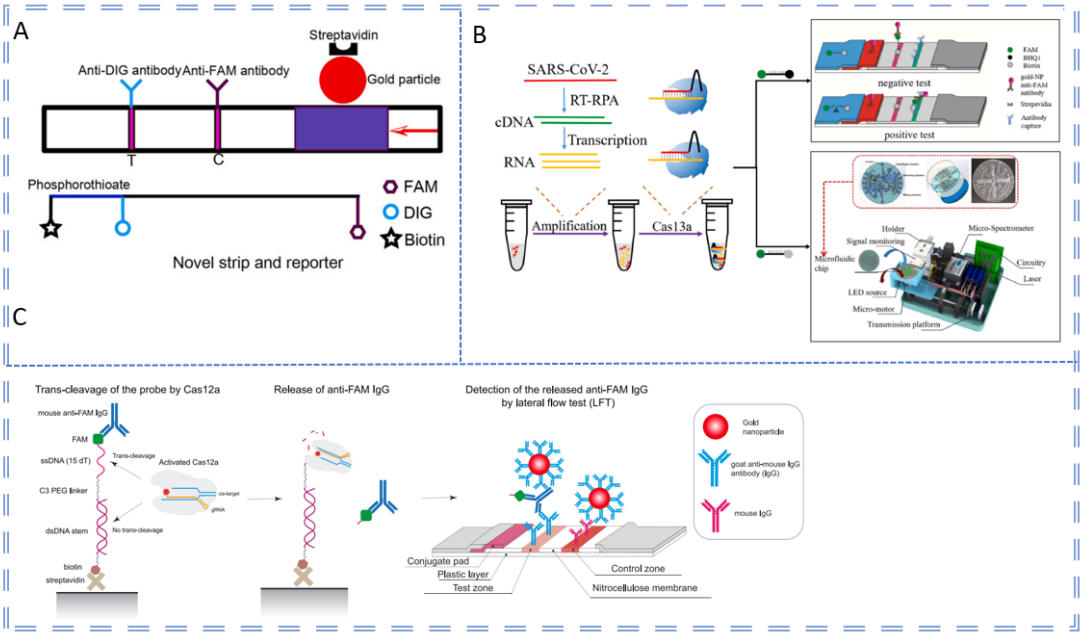


Figure 14 Schematic diagrams for constructed CRISPR/Cas-based lateral flow biosensors. (A) Schematic of the novel strip and reporter. (B) Schematic illustration of the detecting virus strategy combined CRISPR/Cas13a with RPA lateral flow strip. (C) Scheme of Lateral flow biosensor to detect Cas12 trans-cleavage activity.

6 CRISPR/Cas-based detection application

CRISPR/Cas-based detection technology have had extensive applications in biomedicine and food safety field. And in particular for the sensing of nucleic-acid-based biomarkers of infectious and non-infectious diseases and for the detection of

mutations and deletions indicative of genetic diseases. Moreover, the technology has been adapted for the sensing of proteins and small molecules. The basic principle is to release or bind the triggers of the CRISPR/Cas system through the target molecule, resulting in activation or restriction of the accessory cleavage activity in the Cas protein, respectively, and ultimately providing a fluorescent or electrical readout signal, which are used to detect ATP, tobramycin, alkaline phosphatase，ion and so on[73,118-120]。

7 Conclusions and Prospects

CRISPR technology is used in food safety, virus detection, infection and non-infection detection diseases detection. While there are still a few problems which need to be addressed.

First, CRISPR/Cas maybe not be restricted by PAM or PFS sequences in the future; Second, new Cas enzyme can be further identified and modifying existing Cas enzymes. Third, in terms of detection speed, the fastest detection methods (such as SHERLOCKv2, CRISPR-Chip) require at least 15 min in order to improve the reaction speed, search for new stable Cas proteins, and optimize the reaction by adding new ingredients. Last, to fight the CRISPR/Cas system and survive from this adaptive immunity, phages have developed anti-CRISPR proteins (Acrs) to inactivate the CRISPR/Cas system. Therefore, we need to enhance the binding force of crRNA with target DNA and non-specific DNA.

[1] Mojica F J, Díez-Villaseñor C, García-Martínez J, et al.Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements[J].J Mol Evol,2005, 60 (2): 174-82.
[2] Shin J, Miller M, Wang Y C.Recent advances in CRISPR-based systems for the detection of foodborne pathogens[J].Compr Rev Food Sci Food Saf,2022, 21 (3): 3010-3029.
[3] Chen J S, Ma E, Harrington L B, et al.CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity[J].Science,2018, 360 (6387): 436-439.
[4] Abudayyeh O O, Gootenberg J S, Konermann S, et al.C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector[J].Science,2016, 353 (6299): aaf5573.
[5] Koonin E V, Makarova K S, Zhang F.Diversity, classification and evolution of CRISPR-Cas systems[J].Curr Opin Microbiol,2017, 37: 67-78.
[6] Richter H, Rompf J, Wiegel J, et al.Fragmentation of the CRISPR-Cas Type I-B signature protein Cas8b[J].Biochim Biophys Acta Gen Subj,2017, 1861 (11 Pt B): 2993-3000.
[7] Majumdar S, Zhao P, Pfister N T, et al.Three CRISPR-Cas immune effector complexes coexist in Pyrococcus furiosus[J].Rna,2015, 21 (6): 1147-58.
[8] Fozouni P, Son S, Díaz De León Derby M, et al.Amplification-free detection of SARS-CoV-2 with CRISPR-Cas13a and mobile phone microscopy[J].Cell,2021, 184 (2): 323-333.e9.
[9] Park H M, Liu H, Wu J, et al.Extension of the crRNA enhances Cpf1 gene editing in vitro and in vivo[J].Nat Commun,2018, 9 (1): 3313.
[10] Nguyen L T, Smith B M, Jain P K.Enhancement of trans-cleavage activity of Cas12a with engineered crRNA enables amplified nucleic acid detection[J].Nat Commun,2020, 11 (1): 4906.
[11] Ma J Y, Wang S Y, Du Y C, et al."RESET" Effect: Random Extending Sequences Enhance the Trans-Cleavage Activity of CRISPR/Cas12a[J].Anal Chem,2022, 94 (22): 8050-8057.
[12] Ohmichi T, Nakano S, Miyoshi D, et al.Long RNA dangling end has large energetic contribution to duplex stability[J].J Am Chem Soc,2002, 124 (35): 10367-72.
[13] Kara M, Zacharias M.Stabilization of duplex DNA and RNA by dangling ends studied by free energy simulations[J].Biopolymers,2014, 101 (4): 418-27.
[14] Krysler A R, Cromwell C R, Tu T, et al.Guide RNAs containing universal bases enable Cas9/Cas12a recognition of polymorphic sequences[J].Nat Commun,2022, 13 (1): 1617.
[15] Watkins N E, Jr., Santalucia J, Jr.Nearest-neighbor thermodynamics of deoxyinosine pairs in DNA duplexes[J].Nucleic Acids Res,2005, 33 (19): 6258-67.
[16] Amitai G, Sorek R.CRISPR-Cas adaptation: insights into the mechanism of action[J].Nat Rev Microbiol,2016, 14 (2): 67-76.
[17] Xu X, Chemparathy A, Zeng L, et al.Engineered miniature CRISPR-Cas system for mammalian genome regulation and editing[J].Mol Cell,2021, 81 (20): 4333-4345.e4.
[18] Pausch P, Al-Shayeb B, Bisom-Rapp E, et al.CRISPR-CasΦ from huge phages is a hypercompact genome editor[J].Science,2020, 369 (6501): 333-337.
[19] Kato K, Zhou W, Okazaki S, et al.Structure and engineering of the type III-E CRISPR-Cas7-11 effector complex[J].Cell,2022, 185 (13): 2324-2337.e16.
[20] Li Z, Zhao W, Ma S, et al.A chemical-enhanced system for CRISPR-Based nucleic acid detection[J].Biosens Bioelectron,2021, 192: 113493.
[21] Simpson R J.Stabilization of proteins for storage[J].Cold Spring Harb Protoc,2010, 2010 (5): pdb.top79.
[22] Luo G C, Yi T T, Jiang B, et al.Betaine-assisted recombinase polymerase assay with enhanced specificity[J].Anal Biochem,2019, 575: 36-39.
[23] Ralser M, Querfurth R, Warnatz H J, et al.An efficient and economic enhancer mix for PCR[J].Biochem Biophys Res Commun,2006, 347 (3): 747-51.
[24] Wang M, Zhang R, Li J.CRISPR/cas systems redefine nucleic acid detection: Principles and methods[J].Biosens Bioelectron,2020, 165: 112430.
[25] Lu S, Tong X, Han Y, et al.Fast and sensitive detection of SARS-CoV-2 RNA using suboptimal protospacer adjacent motifs for Cas12a[J].Nat Biomed Eng,2022, 6 (3): 286-297.
[26] Chatterjee P, Lee J, Nip L, et al.A Cas9 with PAM recognition for adenine dinucleotides[J].Nat

Commun,2020, 11 (1): 2474.
[27] Rybnicky G A, Fackler N A, Karim A S, et al.Spacer2PAM: A computational framework to guide experimental determination of functional CRISPR-Cas system PAM sequences[J].Nucleic Acids Res,2022, 50 (6): 3523-3534.
[28] Vendrell M, Zhai D, Er J C, et al.Combinatorial strategies in fluorescent probe development[J].Chem Rev,2012, 112 (8): 4391-420.
[29] Yuan L, Lin W, Zheng K, et al.Far-red to near infrared analyte-responsive fluorescent probes based on organic fluorophore platforms for fluorescence imaging[J].Chem Soc Rev,2013, 42 (2): 622-61.
[30] Shen J, Zhou X, Shan Y, et al.Sensitive detection of a bacterial pathogen using allosteric probe-initiated catalysis and CRISPR-Cas13a amplification reaction[J].Nat Commun,2020, 11 (1): 267.
[31] Wang M, Han D, Zhang J, et al.High-fidelity detection of DNA combining the CRISPR/Cas9 system and hairpin probe[J].Biosens Bioelectron,2021, 184: 113212.
[32] Li Y, Mansour H, Watson C J F, et al.Amplified detection of nucleic acids and proteins using an isothermal proximity CRISPR Cas12a assay[J].Chem Sci,2021, 12 (6): 2133-2137.
[33] Moon J, Song J, Jang H, et al.Ligation-free isothermal nucleic acid amplification[J].Biosens Bioelectron,2022, 209: 114256.
[34] Kim H, Lee S, Yoon J, et al.CRISPR/Cas12a collateral cleavage activity for simple and rapid detection of protein/small molecule interaction[J].Biosens Bioelectron,2021, 194: 113587.
[35] Li X, Zhang Y, Liu Q, et al.CRISPR/Cas12a-based fluorescence immunoassay: combination of efficient signal generation with specific molecule recognition[J].Analyst,2022, 147 (17): 3833-3837.
[36] Kim H, Lee S, Yoon J, et al.CRISPR/Cas12a collateral cleavage activity for simple and rapid detection of protein/small molecule interaction[J].Biosens Bioelectron,2021, 194: 113587.
[37] Chen X, Cao G, Wang X, et al.Terminal deoxynucleotidyl transferase induced activators to unlock the trans-cleavage of CRISPR/Cpf 1 (TdT-IU- CRISPR/Cpf 1): An ultrasensitive biosensor for Dam MTase activity detection[J].Biosens Bioelectron,2020, 163: 112271.
[38] Chen X, Cao G, Wang X, et al.Terminal deoxynucleotidyl transferase induced activators to unlock the trans-cleavage of CRISPR/Cpf 1 (TdT-IU- CRISPR/Cpf 1): An ultrasensitive biosensor for Dam MTase activity detection[J].Biosens Bioelectron,2020, 163: 112271.
[39] Liu X H, Li B R, Ying Z M, et al.Small-Molecule-Mediated Split-Aptamer Assembly for Inducible CRISPR-dCas9 Transcription Activation[J].ACS Chem Biol,2022, 17 (7): 1769-1777.
[40] Park S, Zhang Y, Lin S, et al.Advances in microfluidic PCR for point-of-care infectious disease diagnostics[J].Biotechnol Adv,2011, 29 (6): 830-9.
[41] Chen Y, Wu S, Wu H, et al.CRISPR/Cas12a-Based Versatile Method for Checking Quantitative Polymerase Chain Reaction Samples with Cycles of Threshold Values in the Gray Zone[J].ACS Sens,2021, 6 (5): 1963-1970.
[42] Li X, Li R, Yuan Z, et al.One Versatile Cas9-Integrated Single-Tube Duplex Quantitative Real-Time PCR System for Rapid Analysis of CRISPR/Cas-Induced Mutants[J].Anal Chem,2022, 94 (30): 10832-10840.
[43] He C, Lin C, Mo G, et al.Rapid and accurate detection of SARS-CoV-2 mutations using a Cas12a-based sensing platform[J].Biosens Bioelectron,2022, 198: 113857.
[44] Alcántara R, Peñaranda K, Mendoza-Rojas G, et al.UnCovid: A versatile, low-cost, and open-source protocol for SARS-CoV-2 RNA detection[J].STAR Protoc,2021, 2 (4): 100878.
[45] Wang R, Li Y, Pang Y, et al.VIR-CRISPR: Visual in-one-tube ultrafast RT-PCR and CRISPR method for instant SARS-CoV-2 detection[J].Anal Chim Acta,2022, 1212: 339937.
[46] Wang X, Shang X, Huang X.Next-generation pathogen diagnosis with CRISPR/Cas-based detection methods[J].Emerg Microbes Infect,2020, 9 (1): 1682-1691.
[47] Qiu X, Liu X, Ma X, et al.One-Pot Isothermal LAMP-CRISPR-Based Assay for Klebsiella pneumoniae Detection[J].Microbiol Spectr,2022, 10 (4): e0154522.
[48] Shi Y, Kang L, Mu R, et al.CRISPR/Cas12a-Enhanced Loop-Mediated Isothermal Amplification for the Visual Detection of Shigella flexneri[J].Front Bioeng Biotechnol,2022, 10: 845688.

[49] Li C, Chen X, Wen R, et al.Immunocapture Magnetic Beads Enhanced the LAMP-CRISPR/Cas12a Method for the Sensitive, Specific, and Visual Detection of Campylobacter jejuni[J].Biosensors (Basel),2022, 12 (3).
[50] Liu J, Tao D, Chen X, et al.Detection of Four Porcine Enteric Coronaviruses Using CRISPR-Cas12a Combined with Multiplex Reverse Transcriptase Loop-Mediated Isothermal Amplification Assay[J].Viruses,2022, 14 (4).
[51] Tao D, Xiao X, Lan X, et al.An Inexpensive CRISPR-Based Point-of-Care Test for the Identification of Meat Species and Meat Products[J].Genes (Basel),2022, 13 (5).
[52] Lee S Y, Oh S W.Filtration-based LAMP-CRISPR/Cas12a system for the rapid, sensitive and visualized detection of Escherichia coli O157:H7[J].Talanta,2022, 241: 123186.
[53] Selvam K, Najib M A, Khalid M F, et al.RT-LAMP CRISPR-Cas12/13-Based SARS-CoV-2 Detection Methods[J].Diagnostics (Basel),2021, 11 (9).
[54] Zhang Y, Chen M, Liu C, et al.Sensitive and rapid on-site detection of SARS-CoV-2 using a gold nanoparticle-based high-throughput platform coupled with CRISPR/Cas12-assisted RT-LAMP[J].Sens Actuators B Chem,2021, 345: 130411.
[55] Qiu X, Liu X, Ma X, et al.One-Pot Isothermal LAMP-CRISPR-Based Assay for Klebsiella pneumoniae Detection[J].Microbiol Spectr,2022, 10 (4): e0154522.
[56] Zhu Z, Li R, Zhang H, et al.PAM-free loop-mediated isothermal amplification coupled with CRISPR/Cas12a cleavage (Cas-PfLAMP) for rapid detection of rice pathogens[J].Biosens Bioelectron,2022, 204: 114076.
[57] Wu H, Chen Y, Shi Y, et al.Carrying out pseudo dual nucleic acid detection from sample to visual result in a polypropylene bag with CRISPR/Cas12a[J].Biosens Bioelectron,2021, 178: 113001.
[58] Luo X, Xue Y, Ju E, et al.Digital CRISPR/Cas12b-based platform enabled absolute quantification of viral RNA[J].Anal Chim Acta,2022, 1192: 339336.
[59] Ding X, Yin K, Li Z, et al.Sensitive quantitative detection of SARS-CoV-2 in clinical samples using digital warm-start CRISPR assay[J].Biosens Bioelectron,2021, 184: 113218.
[60] Zhang Q, Li J, Li Y, et al.SARS-CoV-2 detection using quantum dot fluorescence immunochromatography combined with isothermal amplification and CRISPR/Cas13a[J].Biosens Bioelectron,2022, 202: 113978.
[61] Chen F E, Lee P W, Trick A Y, et al.Point-of-care CRISPR-Cas-assisted SARS-CoV-2 detection in an automated and portable droplet magnetofluidic device[J].Biosens Bioelectron,2021, 190: 113390.
[62] Ding X, Yin K, Li Z, et al.Ultrasensitive and visual detection of SARS-CoV-2 using all-in-one dual CRISPR-Cas12a assay[J].Nat Commun,2020, 11 (1): 4711.
[63] Hu F, Liu Y, Zhao S, et al.A one-pot CRISPR/Cas13a-based contamination-free biosensor for low-cost and rapid nucleic acid diagnostics[J].Biosens Bioelectron,2022, 202: 113994.
[64] Aman R, Marsic T, Sivakrishna Rao G, et al.iSCAN-V2: A One-Pot RT-RPA-CRISPR/Cas12b Assay for Point-of-Care SARS-CoV-2 Detection[J].Front Bioeng Biotechnol,2021, 9: 800104.
[65] Malcı K, Walls L E, Rios-Solis L.Rational Design of CRISPR/Cas12a-RPA Based One-Pot COVID-19 Detection with Design of Experiments[J].ACS Synth Biol,2022, 11 (4): 1555-1567.
[66] Lei R, Li L, Wu P, et al.RPA/CRISPR/Cas12a-Based On-Site and Rapid Nucleic Acid Detection of Toxoplasma gondii in the Environment[J].ACS Synth Biol,2022, 11 (5): 1772-1781.
[67] Lin M, Yue H, Tian T, et al.Glycerol Additive Boosts 100-fold Sensitivity Enhancement for One-Pot RPA-CRISPR/Cas12a Assay[J].Anal Chem,2022, 94 (23): 8277-8284.
[68] Hu M, Qiu Z, Bi Z, et al.Photocontrolled crRNA activation enables robust CRISPR-Cas12a diagnostics[J].Proc Natl Acad Sci U S A,2022, 119 (26): e2202034119.
[69] Park J S, Hsieh K, Chen L, et al.Digital CRISPR/Cas-Assisted Assay for Rapid and Sensitive Detection of SARS-CoV-2[J].Adv Sci (Weinh),2021, 8 (5): 2003564.
[70] Wu X, Tay J K, Goh C K, et al.Digital CRISPR-based method for the rapid detection and absolute quantification of nucleic acids[J].Biomaterials,2021, 274: 120876.
[71] Wang X, Qin Y, Huang Y, et al.A sensitive and facile microRNA detection based on CRISPR-

Cas12a coupled with strand displacement amplification[J].Spectrochim Acta A Mol Biomol Spectrosc,2022, 279: 121476.
[72] Wang W, Liu J, Wu L A, et al.Nicking enzyme-free strand displacement amplification-assisted CRISPR-Cas-based colorimetric detection of prostate-specific antigen in serum samples[J].Anal Chim Acta,2022, 1195: 339479.
[73] Li D, Ling S, Wu H, et al.CRISPR/Cas12a-based biosensors for ultrasensitive tobramycin detection with single- and double-stranded DNA activators[J].Sensors and Actuators B: Chemical,2022, 355: 131329.
[74] Liu Y, Zhou S, Sun H, et al.Ultrasensitive fluorescent biosensor for detecting CaMV 35S promoter with proximity extension mediated multiple cascade strand displacement amplification and CRISPR/Cpf 1[J].Analytica Chimica Acta,2022, 1215: 339973.
[75] Deng Y, Cao G, Chen X, et al.Ultrasensitive detection of gene-PIK3CA(H1047R) mutation based on cascaded strand displacement amplification and trans-cleavage ability of CRISPR/Cas12a[J].Talanta,2021, 232: 122415.
[76] Zhou S, Sun H, Huo D, et al.A novel methyl-dependent DNA endonuclease GlaI coupling with double cascaded strand displacement amplification and CRISPR/Cas12a for ultra-sensitive detection of DNA methylation[J].Anal Chim Acta,2022, 1212: 339914.
[77] Chi Z, Wu Y, Chen L, et al.CRISPR-Cas14a-integrated strand displacement amplification for rapid and isothermal detection of cholangiocarcinoma associated circulating microRNAs[J].Anal Chim Acta,2022, 1205: 339763.
[78] Wang X, Qin Y, Huang Y, et al.A sensitive and facile microRNA detection based on CRISPR-Cas12a coupled with strand displacement amplification[J].Spectrochim Acta A Mol Biomol Spectrosc,2022, 279: 121476.
[79] Zhang Q, Zhao S, Tian X, et al.Development of a CRISPR-Cas-Based Biosensor for Rapid and Sensitive Detection of 8-Oxoguanine DNA Glycosylase[J].Anal Chem,2022, 94 (4): 2119-2125.
[80] Huang D, Ni D, Fang M, et al.Microfluidic Ruler-Readout and CRISPR Cas12a-Responded Hydrogel-Integrated Paper-Based Analytical Devices (μReaCH-PAD) for Visible Quantitative Point-of-Care Testing of Invasive Fungi[J].Anal Chem,2021, 93 (50): 16965-16973.
[81] Zhuang J, Zhao Z, Lian K, et al.SERS-based CRISPR/Cas assay on microfluidic paper analytical devices for supersensitive detection of pathogenic bacteria in foods[J].Biosens Bioelectron,2022, 207: 114167.
[82] Wu H, Cao X, Meng Y, et al.DropCRISPR: A LAMP-Cas12a based digital method for ultrasensitive detection of nucleic acid[J].Biosens Bioelectron,2022, 211: 114377.
[83] Yu Z, Xu L, Lyu W, et al.Parallel multistep digital analysis SlipChip demonstrated with the quantification of nucleic acid by digital LAMP-CRISPR[J].Lab Chip,2022, 22 (16): 2954-2961.
[84] Xiang X R, Li F, Shang Y T, et al.High-throughput microfluidic strategy based on RAA-CRISPR/Cas13a dual signal amplification for accurate identification of pathogenic Listeria[J].Sensors and Actuators B-Chemical,2022, 358.
[85] Chen Y, Zong N, Ye F, et al.Dual-CRISPR/Cas12a-Assisted RT-RAA for Ultrasensitive SARS-CoV-2 Detection on Automated Centrifugal Microfluidics[J].Anal Chem,2022, 94 (27): 9603-9609.
[86] Cao G, Huo D, Chen X, et al.Automated, portable, and high-throughput fluorescence analyzer (APHF-analyzer) and lateral flow strip based on CRISPR/Cas13a for sensitive and visual detection of SARS-CoV-2[J].Talanta,2022, 248: 123594.
[87] Liu J, Wang H, Zhang L, et al.Sensitive and Rapid Diagnosis of Respiratory Virus Coinfection Using a Microfluidic Chip-Powered CRISPR/Cas12a System[J].Small,2022, 18 (26): e2200854.
[88] Li Z, Ding X, Yin K, et al.Instrument-free, CRISPR-based diagnostics of SARS-CoV-2 using self-contained microfluidic system[J].Biosens Bioelectron,2022, 199: 113865.
[89] Li P, Zeng X, Xue H, et al.CRISPR-Microfluidic Array for Single-Copy DNA mini Barcoding and Rapid Field Species Identification[J].Sensors and Actuators B: Chemical,2022.
[90] Thakku S G, Ackerman C M, Myhrvold C, et al.Multiplexed detection of bacterial nucleic acids using Cas13 in droplet microarrays[J].PNAS Nexus,2022, 1 (1): pgac021.

[91] Ackerman C M, Myhrvold C, Thakku S G, et al.Massively multiplexed nucleic acid detection with Cas13[J].Nature,2020, 582 (7811): 277-282.
[92] Ackerman C M, Myhrvold C, Thakku S G, et al.Massively multiplexed nucleic acid detection with Cas13[J].Nature,2020, 582 (7811): 277-282.
[93] Welch N L, Zhu M, Hua C, et al.Multiplexed CRISPR-based microfluidic platform for clinical testing of respiratory viruses and identification of SARS-CoV-2 variants[J].Nat Med,2022, 28 (5): 1083-1094.
[94] Zhuang J, Zhao Z, Lian K, et al.SERS-based CRISPR/Cas assay on microfluidic paper analytical devices for supersensitive detection of pathogenic bacteria in foods[J].Biosens Bioelectron,2022, 207: 114167.
[95] Cui J Q, Liu F X, Park H, et al.Droplet digital recombinase polymerase amplification (ddRPA) reaction unlocking via picoinjection[J].Biosens Bioelectron,2022, 202: 114019.
[96] Yu Z, Xu L, Lyu W, et al.Parallel multistep digital analysis SlipChip demonstrated with the quantification of nucleic acid by digital LAMP-CRISPR[J].Lab Chip,2022, 22 (16): 2954-2961.
[97] Li C Y, Zheng B, Liu Y H, et al.A boosting upconversion luminescent resonance energy transfer and biomimetic periodic chip integrated CRISPR/Cas12a biosensor for functional DNA regulated transduction of non-nucleic acid targets[J].Biosens Bioelectron,2020, 169: 112650.
[98] Choi J H, Lim J, Shin M, et al.CRISPR-Cas12a-Based Nucleic Acid Amplification-Free DNA Biosensor via Au Nanoparticle-Assisted Metal-Enhanced Fluorescence and Colorimetric Analysis[J].Nano Lett,2021, 21 (1): 693-699.
[99] Yin B, Zhang Q, Xia X, et al.A CRISPR-Cas12a integrated SERS nanoplatform with chimeric DNA/RNA hairpin guide for ultrasensitive nucleic acid detection[J].Theranostics,2022, 12 (13): 5914-5930.
[100] Fu W, Jiang L, Van Geest E P, et al.Sensing at the Surface of Graphene Field-Effect Transistors[J].Adv Mater,2017, 29 (6).
[101] Yang B, Zeng X, Zhang J, et al.Accurate identification of SARS-CoV-2 variant delta using graphene/CRISPR-dCas9 electrochemical biosensor[J].Talanta,2022, 249: 123687.
[102] Yanling - Y, Zhongmin - T, Han - L, et al.- Emerging two-dimensional material nanozymes for theranostic nanomedicine[J].- Biophysics Reports,2021, - 7 (- 3): - 159.
[103] Broto M, Kaminski M M, Adrianus C, et al.Nanozyme-catalysed CRISPR assay for preamplification-free detection of non-coding RNAs[J].Nat Nanotechnol,2022.
[104] X M, X W, Y Q, et al.A novel label-free universal biosensing platform based on CRISPR/Cas12a for biomarker detection[J].Talanta,2022, 251: 123795.
[105] Xie Z, Chen S, Zhang W, et al.A novel fluorescence amplification strategy combining cascade primer exchange reaction with CRISPR/Cas12a system for ultrasensitive detection of RNase H activity[J].Biosens Bioelectron,2022, 206: 114135.
[106] Liu Y, Zhou S, Sun H, et al.Ultrasensitive fluorescent biosensor for detecting CaMV 35S promoter with proximity extension mediated multiple cascade strand displacement amplification and CRISPR/Cpf 1[J].Anal Chim Acta,2022, 1215: 339973.
[107] Zhang Q, Han Y, Li C C, et al.Construction of a dual-functional dumbbell probe-based fluorescent biosensor for cascade amplification detection of miRNAs in lung cancer cells and tissues[J].Chem Commun (Camb),2022, 58 (36): 5538-5541.
[108] Pu J M, Gui M F, Li H B, et al.In situ hand-in-hand DNA tile assembly and CRISPR/Cas12a-based ultrasensitive detection for leukemia diagnosis[J].Sensors and Actuators B-Chemical,2022, 355.
[109] Wu Z, Sun D W, Pu H, et al.A novel fluorescence biosensor based on CRISPR/Cas12a integrated MXenes for detecting Aflatoxin B1[J].Talanta,2022, 252: 123773.
[110] Tao Y, Yi K, Wang H, et al.Metal nanoclusters combined with CRISPR-Cas12a for hepatitis B virus DNA detection[J].Sensors and Actuators B: Chemical,2022, 361: 131711.
[111] Li C-Y, Liu J-X, Liu Y-H, et al.An exceptional and universal DNA walker amplified “one-to-many” CRISPR/Cas12a-mediated fluorescent biosensor for ultrasensitive detection of non-DNA

biomarkers[J].Sensors and Actuators B: Chemical,2022, 361: 131743.
[112] Moon J, Kwon H J, Yong D, et al.Colorimetric Detection of SARS-CoV-2 and Drug-Resistant pH1N1 Using CRISPR/dCas9[J].ACS Sens,2020, 5 (12): 4017-4026.
[113] Wang W, Liu J, Wu L A, et al.Nicking enzyme-free strand displacement amplification-assisted CRISPR-Cas-based colorimetric detection of prostate-specific antigen in serum samples[J].Anal Chim Acta,2022, 1195: 339479.
[114] Tao Y, Yi K, Wang H, et al.CRISPR-Cas12a-regulated DNA adsorption and metallization on MXenes as enhanced enzyme mimics for sensitive colorimetric detection of hepatitis B virus DNA[J].J Colloid Interface Sci,2022, 613: 406-414.
[115] Hu M, Zhu D, Zhou X.M-CDC: Magnetic pull-down-assisted colorimetric method based on the CRISPR/Cas12a system[J].Methods,2022, 203: 259-267.
[116] Gong J, Kan L, Zhang X, et al.An enhanced method for nucleic acid detection with CRISPR-Cas12a using phosphorothioate modified primers and optimized gold-nanopaticle strip[J].Bioact Mater,2021, 6 (12): 4580-4590.
[117] Ivanov A V, Safenkova I V, Zherdev A V, et al.DIRECT(2): A novel platform for a CRISPR-Cas12-based assay comprising universal DNA-IgG probe and a direct lateral flow test[J].Biosens Bioelectron,2022, 208: 114227.
[118] Niu C, Wang C, Li F, et al.Aptamer assisted CRISPR-Cas12a strategy for small molecule diagnostics[J].Biosens Bioelectron,2021, 183: 113196.
[119] Wang X, Zhou S, Chu C, et al.Target-induced transcription amplification to trigger the trans-cleavage activity of CRISPR/Cas13a (TITAC-Cas) for detection of alkaline phosphatase[J].Biosens Bioelectron,2021, 185: 113281.
[120] Xie S, Xu B, Tang R, et al.Kinetics Accelerated CRISPR-Cas12a Enabling Live-Cell Monitoring of Mn(2+) Homeostasis[J].Anal Chem,2022, 94 (28): 10159-10167.