

CRISPR-enabled sensors for rapid monitoring of environmental contaminants

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Abstract

There is increasing attention on the impacts of contaminants on environmental and human health. To better understand the potential threat to ecosystems and human health, biosensing has played an important role in monitoring contaminants and biomarkers. In the past decade, the integration of CRISPR-Cas systems with technologies like microfluidic devices and isothermal amplification methods has paved the way for developing advanced sensors for environmental surveillance. Here we discuss the recent progress of various CRISPR-Cas systems to develop new biosensing devices, ranging from the fundamental mechanisms to their practical applications. We present a comprehensive and critical overview on the current state-of-the-art of CRISPR-Cas-based sensing platforms, including for both nucleic acid and non-nucleic acid contaminants, as well as portable engineered systems for on-site detection. We also provide the prospects of CRISPR-Cas systems for next-generation environmental surveillance, together with emerging technologies such as data science and artificial intelligence.

Keywords: CRISPR-Cas, portable sensors, water contaminants, environmental monitoring

1. Introduction

Detection of contaminants in the environment, especially in water bodies, is crucial for regulating their presence and is also the focal point of environmental issues. Regular monitoring of environmental contaminants can serve as an early warning system of contamination events, such as disease outbreaks, chemical spills and hazardous emissions. Recently, wastewater-based epidemiology (WBE) was used for effective tracking of spatial and temporal trends of the coronavirus disease 2019 (COVID-19) pandemic across entire populations [1, 2]. WBE was performed in a non-invasive, individual-targeted, and privacy-respected manner, serving as a highly efficient tool to guide public health measures. Therefore, monitoring environmental contaminants or biomarkers not only enables quantification of their impact and ensures the safety of aquatic environments, but also aids in preventing and controlling the spread of diseases.

Traditionally, multiple analytical techniques have been utilized for detecting environmental contaminants, including chromatography-mass spectrometry (MS) [3], polymerase chain reaction (PCR) [4], Raman spectroscopy [5], enzyme-linked immunosorbent assay (ELISA) [6], genome sequencing [7], etc. These techniques rely on sophisticated laboratory equipment, laborious sample pretreatment, and well-trained technicians, leading to high-cost and time-consuming procedures. Over the past few decades, sensors have emerged as viable alternatives [8], which are devices that can measure specific physical, electrical, or chemical changes and convert them into usable signal outputs [9, 10]. Biosensors and microfluidic sensors have been utilized for detecting pathogens, heavy metals, organic pollutants, etc., in the environment [11-13]. Although they are capable of rapid analysis, low cost, and on-site testing [14, 15], most of them still have trade-offs in performance metrics. In recent years, the application of clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (CRISPR-Cas) systems in sensors has unlocked tremendous potential in this field [16].

CRISPR-Cas is a naturally occurring adaptive immune system found in bacteria and archaea, comprising CRISPR arrays and Cas proteins [17]. CRISPR portion contains spacer and repeat sequences, which are used to locate and identify target gene fragments, while Cas proteins, acting as nucleases, are responsible for binding to and cleaving target genes. CRISPR-Cas systems have been widely utilized as an innovative and powerful toolbox in genetic editing since 2013 [18]. The discovery of the trans-cleavage activity of Cas enzymes further enabled CRISPR-Cas systems to be used as nucleic acid detection systems [19]. These systems offer single-base resolution along with high sensitivity and specificity, marking a thriving era in CRISPR-based sensors.

To further enhance sensitivity, CRISPR-Cas systems are often integrated with isothermal amplification methods, especially when processing low concentrations of targets in environmental samples. Recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), exponential amplification reaction (EXPAR), and rolling circle amplification (RCA) are favored because they amplify genetic materials rapidly at a constant temperature [20, 21]. Recently, amplification-free CRISPR-based biosensors have also emerged, primarily utilizing gold nanoparticles (AuNPs), gold nanoclusters, nanopores, with different transducers like localized surface plasmon resonance (LSPR), field-effect transistor (FET), or electrochemical transducers [22-25]. For example, A novel LSPR system with AuNPs achieved a 31 nm redshift, and was optimized for SARS-CoV-2 detection via CRISPR-Cas13a, offering high-sensitivity and label-free diagnostics [24]. Integrating CRISPR-Cas sensors into microfluidic devices is a current research focus to maximize their potential for point-of-care testing (POCT) in resource-limited regions [25]. This approach not only capitalizes on

the remarkable sensitivity and specificity of CRISPR-Cas systems but also leverages the simplicity, portability, and cost-effectiveness of microfluidic devices.

Nevertheless, several issues need to be addressed to improve CRISPR-Cas systems and broaden their applicability. First, liquid transfer between amplification and CRISPR-reaction steps reduces accuracy and is time-consuming [26]. This is because additional time is needed for nucleic acid extraction/purification, pre-amplification and CRISPR-reaction. Aerosol contamination and cross-interference between different samples are common challenges during these processes. Even though integrating pre-amplification and CRISPR-reaction into a single step can simplify the workflow, incompatibility between reaction temperatures or variations in reaction components can impact the stability and sensitivity of CRISPR reaction. Secondly, multiplex detection from a single sample is challenging when detecting multiple gene loci simultaneously due to a lack of cleavage-based strategies that can target each gene uniquely [27]. Furthermore, although most CRISPR-based platforms, such as SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) and DETECTR (DNA endonuclease targeted CRISPR trans reporter), offer good sensitivity, their quantitative accuracy is retentively limited [28]. Single nucleotide polymorphism detection is also a challenge sometimes due to gRNA design restrictions. In terms of RNA detection, double stranded RNA detection is still a challenge. Lastly, exploring the feasibility of commercializing CRISPR-based platforms is essential, as cost-effective production and simplified protocols are needed to make these systems accessible on a broader scale.

Overall, CRISPR-Cas systems have been put forward and comprehensively investigated over the past decades due to their remarkable characteristics of high sensitivity, specificity, flexibility, and simplicity, which creates a solid foundation for the advancement of next-generation biosensing technology. This article will review the progress of different CRISPR-Cas systems in biosensing applications and provide a comprehensive comparison. Besides, we provide insights into CRISPR-Cas systems coupled with amplification strategies and the engineering of assays within microfluidic devices for in-field testing. Finally, the applications and future perspectives of CRISPR-Cas systems for detecting various targets in aquatic environments are discussed.

2. CRISPR-Cas enabled sensors for detecting chemical and biological contaminants

CRISPR is derived from the adaptive immune systems of prokaryotes. CRISPR-Cas system comprises a CRISPR array, characterized by periodic repeats and interspersed spacer sequences [29]. Through an adaptive process, CRISPR-Cas machinery scans the genomes of invading phages and induces double-strand DNA breaks (DSBs) at locations upstream of the adjacent protospacer adjacent motifs (PAMs). The resulting DNA fragments are incorporated into the CRISPR array, where they are positioned between direct repeat sequences. These spacer fragments are subsequently transcribed into CRISPR RNA (crRNA), which guides Cas protein to its nucleic acid target for cleavage, ultimately yielding mature crRNA [30].

Capitalizing on the specificity, programmability, and user-friendliness of CRISPR technology, CRISPR-Cas systems have emerged as a versatile tool for genome engineering, transcriptional regulation, and biosensing [31]. Biosensing systems conventionally rely on biomolecules to detect target biomarkers, coupling them with sensors to relay the biomarker-sensor interaction to users. With recognition-triggered cleavage capability, CRISPR-based biosensing is promising in molecular

diagnostics, food safety, and environmental contexts, with sensitivity and specificity while streamlining detection and reducing time [32]. The purview of CRISPR sensing has progressively widened as researchers have pioneered biorecognition elements to transduce non-nucleic acid signals into nucleic acid signals. Nowadays, advances in CRISPR technology development have significantly accelerated sensor design for customizing biological or chemical signal processing. Such CRISPR-based biosensing systems are now extensively used in environmental monitoring, including water quality assessment, and detection of contaminants such as pathogenic microorganisms, heavy metal ions, and antibiotics.

CRISPR-based sensing is inherently suited for nucleic acid detection due to its potential for user-friendly methodologies with minimal sample pretreatment [33, 34]. Despite this, nucleic acids are only a subset of possible biomarkers, prompting inquiry into the applicability of CRISPR technology to non-nucleic acid targets. Adaptor molecules like aptamers, DNA enzymes, and allosteric regulators, offer the potential for CRISPR systems to detect small molecules, proteins, microorganisms, and metal ions [35]. For example, activators, nucleic acid fragments that bind to crRNA and activate CRISPR systems, were integrated into a probe design for target detection. Employing functional DNA (fDNA) such as structure-switching aptamers and DNazymes, an activatable CRISPR-Cas12a system was developed to detect trace biomarkers like Na^+ and ATP, with LODs of 18 nM and 0.37 μM , respectively [36]. Biotin-modified fDNA was also attached to streptavidin-coated magnetic beads (MBs) to fabricate an activatable CRISPR-Cas12 fluorescence system [37]. This approach was demonstrated for Pb^{2+} detection at picomolar (pM) levels and multiplexed miRNA detection at femtomolar (fM) levels.

Beyond their application in biosensing, CRISPR systems have been expanded to encompass chemical sensing, with electrochemical biosensors utilizing reporter genes to bridge CRISPR-Cas systems and electrochemical detection [38, 39]. The detection of targets is signaled by the reporter gene's cleavage status, and an unaltered reporter gene enables a subsequent electrochemical reaction, facilitating a cascade effect between these two systems. Electrochemical biosensors exhibit advantages such as rapid analysis, high selectivity, enhanced sensitivity, streamlined detection, and continuous monitoring capabilities, with potential for integration into portable kits for multiplexed target monitoring [40, 41].

Electrochemical transduction of CRISPR signals is achieved by detecting the trans-cleavage activity induced by the cis cleavage of non-specific ssDNA by Cas effectors. Linear ssDNA reporters are utilized as the sensory interface and immobilized on the electrode surface. The CRISPR-Cas12a-based platform harnessed the redox reaction of methylene blue (MB) for electron transfer [42]. However, the extended distance between linear ssDNA and the electrode surface reduced electron transfer efficiency, leading to diminished electrochemical response. Hairpin DNA was further immobilized on the electrode, reducing electron tunnelling distance and enhancing signal change after Cas12a cleavage. CRISPR-Cas9 has also facilitated electrochemical biosensors, leveraging the single-stranded cleavage activity of mutant Cas9 to generate staggered ends on dsDNA processed by dual Cas9 [43]. These fragments were ligated to a hairpin structure substrate, amplifying the substrate under displacement polymerase catalysis and enhancing signal output.

The development of a non-nucleic acid target analysis strategy involves designing substrate nucleic acids tailored to the target's characteristics or mediating the substrate-target correlation through enzyme activity. By carefully selecting sensing molecules such as aptamers, enzymes, and functional nucleic acids, the analytical capabilities of CRISPR-based sensors can be greatly enhanced. However, substantial efforts and development costs are necessary to integrate biorecognition with electrochemical

or optical signal transduction. CRISPR-Cas systems are expected to act as a programmable biological component in on-site deployment applications.

3. Development of CRISPR-enabled sensors

3.1 Sensing mechanism derived from different Cas enzymes

Within CRISPR-Cas systems, Cas enzymes are characterized by their enzymatic nature and exhibit target-specific cleavage capabilities [44]. The mature crRNA in conjunction with Cas enzymes constitutes a binary complex as a biorecognition element. CRISPR-Cas systems are classified into two principal categories based on the structural and functional attributes of Cas proteins and are further subclassified according to the signature proteins. Class 1 CRISPR-Cas systems are characterized by complexes comprising multiple Cas enzymes, encompassing types I, III, and IV [45]. In contrast, Class 2 CRISPR-Cas systems are defined by single multi-domain Cas enzymes that operate in an RNA-mediated manner, including type II (Cas9), type V (Cas12a, Cas12b, Cas14), and type VI (Cas13a, Cas13b) enzymes [46]. The inherent simplicity and high efficiency of Class 2 Cas enzymes have led to their widespread adoption in sensing applications (Figure 1).

3.1.1 Cas9 enzyme-based CRISPR-Cas sensing systems

Cas9 is a duplex RNA-mediated dsDNA nuclease that cleaves target dsDNA, resulting in the generation of DSBs. Within the CRISPR-Cas9 system, tracrRNA and crRNA are integrated into a single guide RNA (sgRNA) [47]. The recognition and binding of the crRNA/Cas9 complex to target dsDNA depend on the presence of PAM. The PAM interacts with Cas9 through hydrogen bonding, leading to the unwinding of the target dsDNA and promoting the binding of dsDNA to crRNA, thereby stabilizing Cas9 in its active conformation.

CRISPR-Cas9 systems (Figure 1A) are founded on several core principles, including the Cas9-induced unwinding of non-targeted DNA strands for isothermal amplification, the guidance of recombinant split proteins by catalytically inactive Cas9 chaperones, and the Cas9-based destruction of PAM-containing sites. For instance, Pardee et al. [48] developed a paper-based isothermal toehold switching biosensing system by integrating nucleic acid sequence-based amplification (NASBA) with Cas9 enzyme, enabling the differentiation of Zika virus lineages with enhanced specificity at single-base resolution. Huang et al. [49] fabricated the CRISPR-Cas9 triggered isothermal exponential amplification reaction (CASPAREX) by designing individual antisense PAM-presenting oligonucleotides (PAMmers), which detected DNA targets with attomole sensitivity and single-base precision within 1 h.

In addition to utilizing the enzymatic cleavage activity of Cas9 enzyme, nuclease-dead Cas9 (dCas9) with sequence-specific binding capabilities (Figure 1B) are also employed to design CRISPR-Cas sensing systems. dCas9 retains the ability to bind dsDNA and can be fused with various modules, such as cleaved fluorescent proteins or cleavage enzymes. A paired dCas9 reporter system was fabricated, where the N and C termini of firefly luciferase were fused to two dCas9 proteins and guided by a pair of sgRNAs [50]. Furthermore, CRISPR-mediated DNA-fluorescence in situ hybridization (FISH) was reported for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) [51], where dCas9-magnetic nanobead complexes were employed to isolate target DNA from samples in 30 min.

In the Type II CRISPR system, gRNA is composed of crRNA and tracrRNA, both integral to the system's DNA targeting function. A CRISPRa activation device was engineered in bacteria, which relied on crRNA-tracrRNA for activation, inspired by their reported eukaryote-like CRISPRa system [52]. The σ^{54} -dependent promoters-based eukaryote-like CRISPRa system enabled long-distance DNA targeting by combining CRISPRa with dxCas9, enhancing the flexibility of DNA targeting and expanding the dynamic range [53]. This group validated the programmability of crRNA-tracrRNA pairing and developed an Atypical gRNA-activated Transcription Halting Alarm (AGATHA) biosensor to detect severe acute respiratory syndrome virus 2 (SARS-CoV-2) RNA in vitro [54]. This approach can be adapted into standardized and modular toolkits, which will enable the construction of synthetic biological biosensors for broad-scale deployment [55].

3.1.2 Cas12 enzyme-based CRISPR-Cas sensing systems

Cas12 is a single RNA-mediated DNA nuclease, with Cas12a being the most prevalent isoform. Different from Cas9, Cas12a exhibits a broader range of target recognition, encompassing both dsDNA and ssDNA (Figure 1C). In CRISPR-Cas12 systems [45], PAM is necessary for dsDNA recognition, whereas PAM is not required for ssDNA recognition. The PAM site is characterized by a high abundance of thymine (T) bases, thereby expanding the target gene repertoire beyond the G-rich PAMs used by Cas9. Furthermore, Cas12a exhibits not only cis-cleavage activity against specific target genes but also trans-cleavage activity against non-specific ssDNA.

The first Cas12a-based sensing system reported in 2018 was DETECTR for detecting viruses and differentiating human papillomavirus genotypes [56]. Cas12a was directed to the dsDNA target via complementary crRNA, leading to the incidental cleavage of a short ssDNA reporter. Target recognition and reporter cleavage resulted in the dissociation of the quencher from the fluorophore, thus generating a fluorescent signal. In another sensing system HOLMES (one-hour low-cost multipurpose highly efficient system), Cas12a/crRNA formed a target DNA-RNA hybrid complex in the presence of target DNA, leading to trans-cleavage of non-targeted ssDNA [57]. By using LbCas12a preamplification, HOLMES could distinguish viral genotypes and human single nucleotide polymorphisms (SNPs) with attomole sensitivity in 1 h.

Cas12b shares the same DNA trans-cleaving activity as Cas12a and exhibits a broader temperature range for trans-cleavage reaction. Cas12b exhibits higher enzymatic activity against dsDNA and demonstrates greater heat resistance than Cas12a. Cas12b was employed to develop an enhanced version of HOLMES (HOLMESv2), which represents a one-pot nucleic acid detection system capable of DNA quantification [58]. HOLMESv2 specifically identified SNPs and detected viral RNA, human cell mRNA, and DNA methylation by integrating LAMP. Similarly, the ssDNA reporters in DETECTR and HOLMES can be labeled with paired fluorophores/quenchers for fluorescence detection or combined with colorimetric strategies for visual readout.

3.1.3 Cas13 enzyme-based CRISPR-Cas sensing systems

Cas13 is a single RNA-mediated RNA-targeting nuclease that directly cleaves RNA, enabling transcriptional-level gene editing (Figure 1D). Different from Cas9 and Cas12, Cas13a exhibits two distinct RNase activities: one for processing crRNA and the other for hydrolyzing target ssRNA [59]. Following cleavage of the captured target, the Cas13-crRNA complex undergoes side-branch cleavage,

leading to the cleavage of non-specific ssRNA. This cleavage activity is harnessed for signal reporting by employing ssRNA-quenched reporters.

Cas13-based sensing systems are reported for nucleic acid detection within a pool of transcripts. The first Cas13a-based sensing system termed SHERLOCK was developed by Gootenberg's group [60]. SHERLOCK possesses biosensing capabilities that allow for the detection of DNA and RNA targets with single-base discrimination. SHERLOCK was demonstrated to detect specific strains of Zika and Dengue viruses, differentiate between different pathogenic bacteria, and identify tumor DNA mutations. Subsequently, the development of HUDSON (heating unextracted diagnostic samples to obliterate nucleases) enabled SHERLOCK to detect viral hemorrhagic fever nucleic acids directly from biological fluids at concentrations as low as 1 copy/ μ L, eliminating the need for nucleic acid extraction and purification.

To enhance diagnostic capabilities, Cas13b-based sensing system SHERLOCKv2 was fabricated to detect Zika and dengue viruses through test strips and identify mutations [61]. By capitalizing on the distinct specificities of various Cas enzymes (including Cas12 and Cas13), SHERLOCKv2 facilitated multiplexed detection of four targets within a single reaction. Sensitivity is enhanced through the incorporation of the auxiliary CRISPR-associated enzyme Csm6. Csm6 is integrated with crRNA-guided Cas13 enzymes to enable direct analysis of biological samples. SHERLOCKv2 enabled quantitative multiplexed sensing at zeptomolar (zM) concentrations and introduced lateral flow readout. Additionally, Joung et al. [62] developed STOPCovid (SHERLOCK testing in one pot) by introducing improved gRNA, tailoring taurine to enhance reaction kinetics and incorporating flow reading.

3.1.4 Cas14 enzyme-based CRISPR-Cas sensing systems

Cas14 exhibits specific targeting activity on ssDNA substrates without the need for PAM (Figure 1E). Cas14 is a small protein that is half the size of other Cas proteins, which are classified into three subtypes (Cas14 a-c) [57]. The cis cleavage of target ssDNA by Cas14 leads to indiscriminate cleavage of trans ssDNA, mirroring the behavior of Cas13 and Cas12 for RNA and dsDNA, respectively. Cas14 is not constrained by PAM and can recognize ssDNA targets, thereby offering an advantage in the development of novel sensing systems.

The target-dependent, nonspecific DNase activity of Cas14 was harnessed as a high-fidelity DNA detection system (Cas14-DETECTR). Compared with Cas12-DETECTR, Cas14-DETECTR demonstrated greater specificity and activity, facilitating high-fidelity detection of DNA SNPs [63]. CRISPR-Cas14a system was integrated with cyclic miRNA-triggered SDA for miRNA detection, where Cas14a was effectively activated by SDA amplicons, resulting in a sensitivity 2.86 times higher than Cas12a. Wu et al. [64] comprehensively elucidated the DNA recognition and cleavage mechanism of Cas14, which consisted of 422 amino acids, thereby providing a theoretical foundation for the subsequent advancement of molecular technology.

CRISPR-Cas-based sensing systems exhibit advantages, including sensitivity to fM or aM concentrations and ultra-high resolution of single-base differences. Both Cas12- and Cas13-based sensing systems depend on the collateral cleavage activities of Cas enzymes, with Cas12 cleaving ssDNA reporters and Cas13 cleaving ssRNA reporters. These systems utilize reporter molecules labeled with fluorescence and quenchers, with reporter dissociation detectable through colorimetric changes. Cas13's ability to cleave target sequences without the need for PAM broadens its application scope compared to Cas12. In addition, Cas14 can differentiate between individual SNPs responsible for eye

color, a capability not shared by Cas12a. This suggests that the evolutionary insights gleaned from Cas enzymes, in conjunction with molecular techniques, will empower biochemists to utilize the inherent modularity of Cas enzymes to enhance their sensing applications.

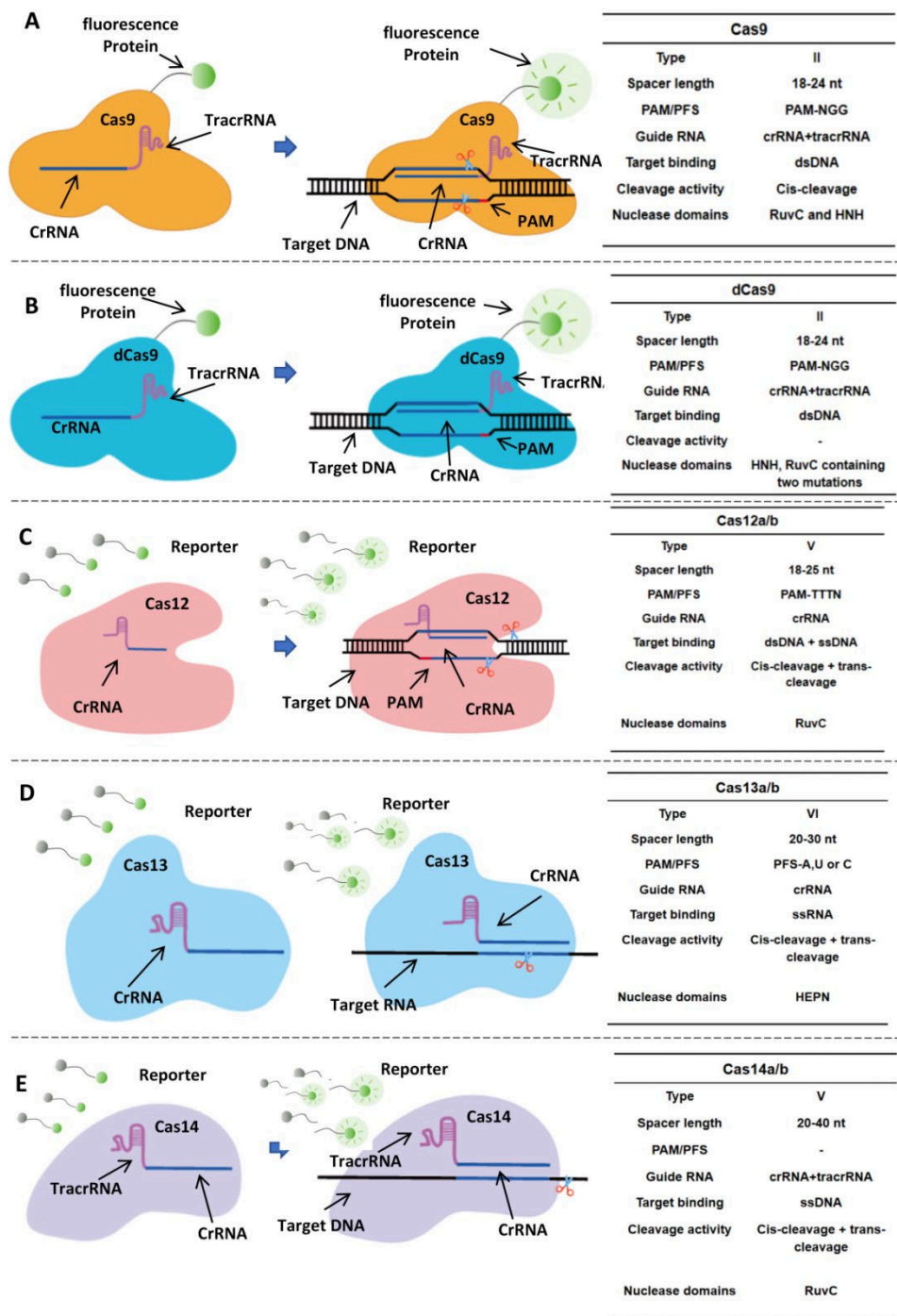


Figure 1. Different Cas enzymes for sensing, including (A) Cas9 enzyme, (B) dCas9 enzyme, (C) Cas12 enzyme, (D) Cas13 enzyme, and (E) Cas14 enzyme.

3.2 CRISPR-Cas system coupled with different amplification strategy

3.2.1 Isothermal amplification methods coupled with CRISPR-Cas system

Nucleic acid-based diagnostics has relied on PCR as the gold-standard method for nucleic acid amplification and detection. Many variants of PCR have been developed since its introduction in 1985, including Real-time PCR, Reverse PCR, Quantitative PCR, as well as digital and droplet digital forms [65]. These advancements have propelled PCR-based assays to unparalleled levels of analytical performance, with LODs as low as 1-10 target molecules and the capacity of multiplexed detection [66]. Whilst PCR machines are commonplace in laboratories worldwide, the rise in laboratory-free diagnostics and on-site monitoring of environmental contaminants has given rise to isothermal amplification techniques capable of amplifying and detecting nucleic acids at a constant temperature, thus circumventing the need for thermocyclers.

The most widely used techniques are LAMP, NASBA, RPA, RCA, SDA, and Hybridization chain reaction (HCR). While these techniques offer sensitivities that match and often surpass that of PCR, they are prone to false positive results and non-specific primer binding [67]. On the other hand, CRISPR-Cas-based techniques display increased levels of specificity, which is attributed to the target-specific guide RNA which can distinguish small differences in target sequence, even at SNP levels [68]. The concurrent emergence of CRISPR-Cas systems and isothermal amplification methods has thus paved the way for developing susceptible and specific nucleic acid detection technologies, capable of rapid and precise molecular diagnostics.

The first published methodology to combine isothermal amplification with CRISPR-Cas came from Feng Zhang's group in 2017 [19] and presented a CRISPR-based diagnostic platform termed SHERLOCK. This platform utilized an ortholog of Cas13a from *Leptotrichia wadei* (LwCas13a) capable of RNA-guided RNase activity. The LOD of 50 fM fell short of the necessary sensitivities for diagnostic application (aM levels). RPA, known for its sensitivity and compatibility with subsequent transcription by T7 RNA polymerase, was thus selected as the ideal isothermal amplification technique for LwCas13a detection. SHERLOCK proved successful in clinical isolates, further demonstrating its capacity for real-world applications. Furthermore, the assay proved compatible with lyophilization of RPA reagents and paper spotting of Cas13a detection, underscoring its potential for field deployment in resource-limited settings. In parallel to the advancements in SHERLOCK, Jennifer Doudna's group reported the development of a rapid one-pot methodology using Cas12a and isothermal amplification, termed DETECTR [69].

Since Doudna's and Zhang's pioneering work, many other methodologies have been reported that combine isothermal amplification with CRISPR-Cas systems to produce ultrasensitive nucleic acid detection. Guo et al. [70] utilized RPA and CRISPR-Cas12 to detect *Phytophthora sojae* (*P. sojae*), with genomic DNA levels as little as 10 pg/ μ L in 20 min. RPA was also used by Xiong et al. [71] to create a one-pot RPA-CRISPR-Cas13a platform for detecting both African swine fever virus and Capripoxi virus. To surpass incompatibility and prevent cross-contamination, the tube was spun, pulling down CRISPR reagents from the lid to mix with RPA amplicons at the bottom of the tube. More recently, Miao et al. [72] developed a one-pot RPA-CRISPR-Cas13a assay for Nipah virus detection utilizing lyophilized reagents with a LOD of 103 cp/ μ L. To step away from lateral flow and fluorescent detection strategies, RPA has also been used to create a bioluminescent nucleic acid sensor platform (LUNAS) utilizing RPA amplification and dCas9-mediated split NanoLuc complementation. Bioluminescent detection was performed with an ordinary digital camera, rendering this readout technique particularly appealing for point-of-care (POC) and point-of-use (POU) testing.

Aside from RPA, LAMP has also been incorporated into CRISPR-Cas-based detection. Li et al. [73] developed a LAMP-CRISPR-Cas12a technique with immunocapture magnetic beads (ICB) to enhance sensitivity. *Campylobacter jejuni* (*C. jejuni*) cells were captured by ICB, and the LAMP products were mixed with CRISPR-Cas12a reagents for visual detection under LED blue light, with a LOD of 8 CFU/mL in 70 min. Ortiz-Cartagena et al. [74] also incorporated reverse-transcription loop-mediated isothermal amplification (RT-LAMP) into their CRISPR-Cas13a system. This method detected SARS-COV-2 from nasopharyngeal samples without RNA extraction, greatly simplifying protocols whilst achieving specificity (100%) and sensitivity (83%). Another Cas12a assisted ligation-initiated LAMP platform for ultrasensitive microRNA detection was developed by Zhang et al. [75], which eliminated the effects of non-specific amplification and false positive results.

HCR, RCA and SDA, although less widely used than LAMP and RPA, have also been incorporated into CRISPR-Cas systems to detect a variety of organisms and molecules such as miRNA, ATP, AFM1, Alpha-fetoprotein, *Escherichia coli* (*E. coli*) and *Salmonella* amongst others [76-81]. Overall, integrating CRISPR-Cas systems with isothermal amplification strategy represents a significant advancement in molecular diagnostics. The combination of these technologies has enabled attomolar (aM) and single molecule sensitivities, with the collateral cleavage activity being readily detectable via lateral flow strips and visual fluorescence. Isothermal amplification techniques do not require bulky and costly equipment, rendering the detection systems suitable for POC and POU applications. Moreover, the development of one-pot detection assays and lyophilization assays are pivotal for their broader application. By eliminating the need for cold storage of reagents, these advancements not only reduce logistical challenges but also contribute to shorter times, lowered costs, and simplified procedures, further enhancing the feasibility and accessibility of ultrasensitive molecular diagnostics.

3.2.2 Amplification-free CRISPR-Cas detection methods

CRISPR-Cas systems have demonstrated considerable potential in molecular diagnostics, and they are inherently linked to the amplification processes as delineated in the previous section. The main reason is that the sensitivity of CRISPR-Cas system is less desirable than that of other nucleic acid testing [82]. Therefore, the strategy is to increase target concentration via amplification before CRISPR-Cas identification, as in the case of DETECTR and SHERLOCK platforms, which used LAMP and RPA, respectively [83, 84]. Although amplification can enhance sensitivity, it also introduces complexities affecting effectiveness and applicability, especially in clinical diagnostics, such as the risk of aerosol contamination leading to cross-contamination [85]. Despite attempts being made to mitigate this through single-tube configurations, this often results in compromised reaction efficiency [86]. Non-specific amplification and primer-related interferences also compromise specificity, sensitivity, and accuracy. Moreover, the added steps prolong the overall analysis time, which is not ideal in urgent situations.

In recent years, increasing studies have been conducted on CRISPR-based applications, with a significant expansion in amplification-free CRISPR-based diagnostics. The first main tactic focuses on improving the two essential elements of CRISPR-Cas systems, crRNAs and output reporter. Regardless of which CRISPR-Cas system is used, they all rely on the sequence-specific targeting ability of crRNAs to generate subsequent cleavage activity. Studies reveal that the length and sequence composition of crRNAs can have a substantial impact on effectiveness. For example, crRNAs with spacer lengths of 20 nucleotides or more are essential for effective target cleavage by Cas13a, as the efficiency is greatly diminished with shorter spacers [87]. Furthermore, using different crRNAs, each targeting a distinct

region of the same nucleic acid may synergistically enhance the cleavage activity of Cas nucleases, thereby increasing the signal output [88].

The performance of CRISPR-Cas-based assays are directly impacted by signal reporters. In order to obtain greater sensitivity and shorter reaction time, recent developments have also concentrated on modifying the characteristics of these reporters. It is demonstrated that the catalytic effectiveness of Cas enzymes may be increased by varying the length of the reporter and adding structural components like hairpin DNA [89]. Fluorescence signals have been further improved by novel designs, such as dual-labeled ssDNA probes that split apart upon target identification [90]. Another significant advancement is using RNA aptamers, which bind to certain dyes and significantly increase signal-to-background ratios upon target identification [91].

Digital assays, which divide responses into several micro-units, have become a powerful tool in biomedical analysis, and the integration of CRISPR-Cas systems with digital droplet technology has led to the development of highly sensitive and amplification-free biosensing platforms. Tian et al. [92] developed an ultra-localized Cas13a assay using picolitre-sized droplets for RNA detection. Each droplet emits a fluorescence signal if RNA is present, enabling absolute RNA quantification — achieving sensitivity levels over 10,000 times greater than conventional assays. Similarly, Yue et al. [93] adapted this microfluidic approach for Cas12a, known for its lower efficiency compared to Cas13a. They enhanced sensitivity significantly by using multiple crRNAs, with DNA detection levels as low as 100 fM, a more than 50-fold improvement over previous Cas12a assays.

In addition to detecting fluorescence intensity changes caused by reporter cleavage, different signal output modes were designed based on CRISPR-Cas-mediated biosensing strategies. The current variation and conversion of Raman scattering intensities is another amplification-free strategy. The graphene-based field effect transistor (gFET), was the first biosensing platform that introduced an electronic readout in CRISPR-Cas-based detection [94, 95]. According to the hybridization of the target DNA with the sgRNA molecules immobilized on the gFET electrode, the negative charge of target DNA caused the ion concentration change to switch on the gate, leading to a higher current response. Hajian et al. [22] developed CRISPR/dCas9-based gFET biosensors to detect target sequences within intact genomic material, and digitally detected target DNA as low as 1.7 fM within 15 min.

CRISPR-Cas-based electrochemical biosensing platforms are another strategy for amplification-free detection. The first CRISPR-Cas12a electrochemical biosensor was employed by placing an electrochemical tag MB on a gold electrode to form a thiolated-ssDNA-MB conjugate [96]. The MB probes were cleaved by the activated CRISPR-Cas12a, leading to a decrease in the current peak. Based on this method, researchers developed a series of detection systems by altering the electrochemical tags to improve sensitivity, such as using an optimized hairpin DNA probe instead of linear ssDNA [97], using multiple MB tags conjugated to streptavidin-AuNPs as a whole to link Au electrode via linear SH-ssDNA-Biotin [98]. The induced CRISPR-Cas system enhanced the sensitivity and accuracy of electrochemical DNA sensor to the fM level, indicating its capability of single mutation detection [99].

Besides, connecting Surface-enhanced Raman spectroscopy (SERS) with CRISPR-Cas systems can also develop amplification-free detection. It has been proved that Cas12a combined with Raman spectroscopy can detect multiple 1 aM nucleic acids within 20 min. Researchers used a similar method to develop a SERS-based CRISPR-Cas12a platform to identify SARS-CoV-2 from clinical samples [100]. Based on this, Pang et al. [101] focused on portability improvement and set up a SERS-based CRISPR-Cas12a lateral flow device with a LOD of 0.3 fM. Compared with the first SERS-based

CRISPR-Cas platform that achieved single molecule detection, the LOD of the latter two is fM level. However, they are portable signal read-out systems suitable for POCT in low-resource areas.

Above all, the main challenges resulting in the LOD of amplification-free CRISPR-Cas systems are commonly at pM levels due to the lack of the most suitable crRNAs, high-efficient probes, flexible signal output modes and the restriction of trans-cleavage catalytic efficiency of Cas proteins. Researchers have improved sensitivity by strategies such as optimizing engineered crRNAs and signal reporters to improve the signal-to-background ratio and connecting these systems with practicable modifications, and ultrasensitive digital platforms to achieve finer detection resolution and greater signal responses. Nevertheless, certain obstacles remain to be solved before universal tools for real-life applications are introduced. Firstly, a specific PAM sequence is crucial for most of the Cas nucleases' activity, and preamplification of target molecules can introduce PAM. Without preamplification, the limitation of target sequence selection occurs in many cases. Moreover, without preamplification, researchers cannot convert target type to achieve CRISPR-Cas biosensors to detect various analytes, including nucleic acids and biological molecules, causing the limited range of target options and compromised universality and flexibility of detection.

3.3 Engineered CRISPR/Cas Assay for in field testing

In recent years, CRISPR-Cas systems have gained significant momentum in molecular diagnostics. However, challenges such as elevated costs, complexity of assay procedures and lack of quantitative measurements have highlighted the need for improved methodologies. As a result, the integration of microfluidics has emerged as a promising approach to enhance the practicality of CRISPR-Cas systems [102]. Based on the huge advantages of microfluidic devices such as portability, miniaturization, multiplexed detection, rapid assay times, low fabrication costs, and low reagent volumes, this section explores the synergistic potential of combining microfluidics with CRISPR-Cas systems to advance molecular diagnostic capabilities.

3.3.1 Integration with microfluidic and paper-based devices

Microfluidic systems are characterized by their capability to manipulate small volumes of fluids, ranging from μL to fL, using microchannels embedded or etched onto a hydrophobic substrate. The flow of liquid through microchannels can occur passively through capillary forces or be achieved actively via micropumps and valves. This controlled liquid flow enables the transport, mixing and separation of reagents in sequential order, allowing for the miniaturization and automatization of various laboratory processes in a single device.

Microfluidic systems used for sensing applications are mainly classified into micro-total analytical systems (μTAS) and microfluidic paper-based analytical devices (μPADs). μTAS are manufactured using materials such as polydimethylsiloxane (PMS), glass, silicon, and other polymers, and connected to external pumps, droplet generators, microheaters and injectors. On the other hand, μPADs are manufactured by hydrophobic coating, photoresist coating, chemical treatment, or wax printing on a paper substrate. The liquid flow is via passive capillary action, and a single device contains all the sensing components [103].

Paper-based microfluidic sensors are particularly well-suited for laboratory-free settings as they do not require external units. Gayet et al. [104] developed a μPAD that used Cas12a-sensitive hydrogels for

Ebola diagnosis, where a polyacrylamide-DNA (PA-DNA) hydrogel containing ssDNA crosslinkers was designed. Yin et al. [105] developed an autonomous lab-on-paper platform for SARS-CoV-2 diagnosis. To achieve simultaneous detection of nucleoprotein (N) gene and spike (S) gene, reverse transcription-recombinase polymerase amplification (RT-RPA) was combined with CRISPR-Cas12a detection. The lab-on-paper platform consisted of a 3D-printed RPA amplification reactor and a paper-based CRISPR-Cas12a detection chamber with pre-loaded lyophilized reagents.

Zhuang et al. [106] developed a SERS-based CRISPR-Cas assay on a μ PAD that integrated RPA for detecting *Salmonella typhimurium* (*S. typhimurium*) with a LOD of 3-4 CFU/mL within 45 min (Figure 2A). The assay utilized a thiolated ssDNA-conjugated gold nano-star reporter probe that was cleaved to produce a dispersed colloidal solution upon activation of Cas12a in the presence of target DNA. In the absence of target molecules, the nanoprobe aggregated when subjected to mild centrifugation, generating SERS signals of different intensities when the target was present or absent. More recently, Zhang et al. [107] reported the use of a paper-based CRISPR-Cas12a microfluidic biosensor for Pb²⁺ detection, demonstrating its potential for detection purposes other than nucleic acid-based diagnosis (Figure 2B). Overall, CRISPR-based μ PADs greatly simplify the operational procedures and costs associated with tube-based CRISPR-Cas assays and, most importantly, provide laboratory-free POCT.

Some limitations still exist, however, including limited multiplexed capabilities due to channel design, lack of absolute quantification and incomplete automation. Moreover, μ PADs are often open systems that can produce cross or environmental contamination via aerosols, giving rise to false positive results. On the other hand, closed microfluidic systems, such as centrifugal microfluidic chips, offer a closed system for contamination-free testing. The controlled and precise flow of reagents driven by centrifugal forces enables the integration of various modules, such as preloading, heating, splitting, and detection, automating the operational procedures [108].

In 2021, Wu et al. [109] reported the development of a reversible valve-assisted chip coupling with Cas12a to detect *Vibrio parahaemolyticus* (*V. parahaemolyticus*) (Figure 2C). The microchip consisted of a polymethylmethacrylate (PMMA) plate, PMMA tape, reaction tube, four reservoirs, three rotary valves, a silica membrane, a cylindrical internal screw pipe and a buckle. The chip was used to extract nucleic acids by adding lysis and wash reagents to two individual reservoirs and centrifuged clockwise to pass the sample through the silica membrane and onto the waste reservoir. The reaction tube contained lyophilized LAMP reagents, which were dissolved once the elute was centrifuged into the reaction tube. After 30 min of isothermal amplification, the CRISPR-Cas12a reagents were added into the tube and the results were visualized with fluorescence, achieving sensitivities of 30 copies/reaction.

While centrifugation-based microfluidics offer process automation involving preamplification, sample transfer and CRISPR-Cas-based detection, they cannot offer absolute quantification results. Digital microfluidic systems that can separate samples into partitions or droplets, encapsulating 0 or 1 copy of nucleic acid, can be used to apply Poisson distribution and achieve quantification. Ackerman et al. [110] developed a combinatorial arrayed reaction for multiplexed pathogen detection termed CARMEN (Figure 2D). The chip was used to rearrange the reagents into thousands of microwells, each representing a unique combination of sample and CRISPR reagents. The collateral cleavage activity of Cas13a was detected via colour barcodes, allowing the differentiation of 169 human viruses in one test with 8-10 h. The CARMEN assay was later improved, giving rise to mCARMEN, which introduced a commercially available fluidic circuit microarray with 100% sensitivity and a LOD of 102 copies/ μ L [111].

Tian et al. [92] reported an ultra-localized Cas13 assay for amplification-free RNA diagnostics (Figure 2E). The entire workflow was conducted in a single isothermal step, implementing a syringe-driven microfluidic droplet chip and standard fluorescence microscopy. The sample was pipetted into the sample inlet, generating highly uniform microdroplets that were then self-assembled into a droplet array. Fluorescence from the droplet array was detected using a standard fluorescence microscope. The assay achieved >10000-fold increase in sensitivity compared to bulk Cas13a assays and enabled absolute RNA quantification.

Wu et al. [112] developed a LAMP-Cas12a-based digital technique, termed drop CRISPR, for ultrasensitive detection at fM level of *S. typhimurium invA* gene. To avoid the problems associated with reaction temperature, a microfluidic device capable of partitioning LAMP reagents into stable droplets was first used to generate LAMP amplicons. A picoinjector was then used to actively introduce CRISPR-Cas12a reagents into droplets after LAMP reaction. More recently, various droplet microfluidic devices have been developed to achieve ultrasensitive, multiplexed and quantification capabilities for CRISPR-Cas-based detection.

Overall, microfluidics for CRISPR-Cas-based detection presents a state-of-the-art approach for sensitive and rapid diagnostics. Microfluidics encompasses diverse technologies, each presenting distinct advantages and limitations. Paper-based microfluidics provide a cost-effective, easy-to-operate alternative to conventional techniques, particularly suited for POC and POU detection in resource-limited settings, as they typically do not require additional components. Centrifugation microfluidics, on the other hand, offer closed systems that enable contamination-free and automated detection procedures, enhancing assay reliability. Lastly, droplet microfluidics leverages low sample volumes to significantly increase sensitivity and is ideal for quantification purposes. Each of these microfluidic approaches contributes uniquely to the advancement of diagnostic technologies, catering to specific needs and applications within the field.

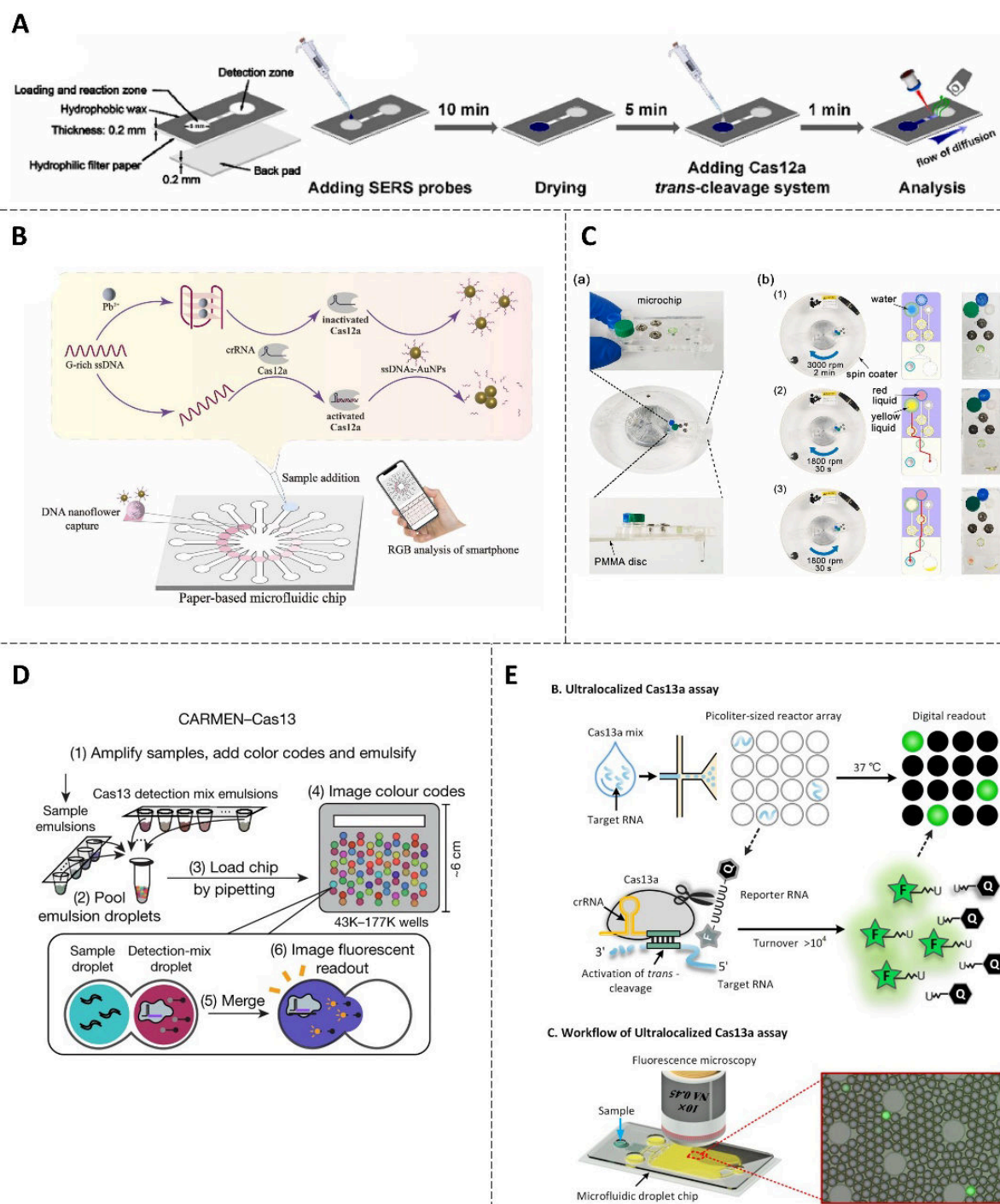


Figure 2. Microfluidic and paper analytical devices for environmental contaminants. (A) Illustration of the μ PAD and the operation of the SERS-based CRISPR-Cas μ PAD. Adapted with permission from Ref. [106]. (B) Illustration of a paper-based microfluidic chip for colorimetric analysis of Pb²⁺. Adapted with permission from Ref. [107]. (C) Photos of a microchip and the rotation of the microchip with a spin coater. Adapted with permission from Ref. [109]. (D) Illustration of the CARMEN-Cas13 workflow. Adapted with permission from Ref. [110]. (E) Schematic illustration of the ultra-localized Cas13 assay and the workflow. Adapted with permission from Ref. [92].

3.3.2 Lateral flow devices

Among the various techniques employed in microfluidic devices, lateral flow assays (LFAs) have gained significant attention due to their easy handling, rapidity, and cost-effectiveness. LFAs were first created in the 1970s to detect biomolecules and first launched commercially in 1984. LFAs typically consist of an absorbent pad, a sample pad, a conjugate pad, and a reaction membrane with a test line and a control line supported by a plastic backing card [113]. After loading the droplet to the sample pad, it will flow through the device by capillary force and the target analytes will bind to the capture antibodies in the test line to form a colour band. By observing the colour change, qualitative or semiquantitative results could be obtained by the optical signal in a short period for on-site analysis [114-116].

LFAs can be integrated with CRISPR technologies to engineer biosensors capable of performing POC and POU detection. More recently, CRISPR-Cas12a has been used in conjunction with the multi-channel lateral flow test strip to monitor SARS-COV-2 in wastewater [114]. To embed CRISPR-Cas12a with LFA, lateral flow strips were incorporated with μ PAD and LAMP device as an integrated platform [117]. It combines the workflow from sample processing, lysis, nucleic acid extraction, enrichment, and amplification, to readout at the closed lateral flow unit, which was specially designed to impede aerosol dispersion and minimize cross-contamination. After amplicon recognition, ssDNA probes modified with reporter molecules could help to implement visualization via lateral flow strips due to the trans-cleavage of CRISPR-Cas12a. Blind experiments proved high sensitivity and selectivity of this platform on wastewater analysis, demonstrating it as a promising tool for rapid in-field wastewater surveillance.

3.3.3 Electrokinetic devices

Electrokinetic techniques counter the drawback of recent micro/nanoscale sensors as an enrichment technique in its speed and maximum enrichment, reliance on biomolecules' electric charges and not the biochemical properties, thus having more comprehensive coverage for several biomolecules, simple technology and ease of application for users, constant pH gradients and electrolyte system, operational in fluid mechanics leading to ease of engineering. Different from conventional nucleic acid extraction, electrokinetic technologies need no absorption, washing, and elution steps but concentrate nucleic acids through the motion and aggregation of charged nucleic acids in electrokinetic-driven chips [28].

Ramachandran et al. [118] demonstrated the isotacophoresis (ITP) technique on a microfluidic chip to extract target DNA from nasopharyngeal samples of COVID-19 patients and healthy controls. Additionally, they applied an ITP electrical field gradient to exert an enzymatic control of CRISPR-Cas12 system to detect SARS-CoV-2 RNA from nasopharyngeal samples within 35 min. They found an increase in specificity of the ITP-CRISPR assay and reduced use of reagents (0.2 μ L of reagents on chip for CRISPR reactions as opposed to 100 times higher consumption of reagent volumes when CRISPR tool was solely used) and a LOD of 10 copies/ μ L. The drawback was observed in the low sample volume of 10 μ L, which reduced sensitivity and intermediate off-chip manual steps for sample lysis. Bruch et al. [119] developed a CRISPR-powered electrochemical biosensor for amplification-free miRNA diagnostics. They divided a single channel of an electrochemical microfluidic chip into subsections by hydrophobic stopping barriers and used a 'stop-flow protocol' to control liquid flows.

4. CRISPR-Cas systems for the detection of nucleic acid and non-nucleic acid targets in real water system

4.1 Nucleic acid-based microbiological detection

Microorganisms, viruses, and antibiotic resistance genes (ARGs) are the three biomarkers in the environment that can be tracked through the specific recognition ability of CRISPR-Cas-based technology for nucleic acid detection. Nowadays, several CRISPR-Cas-based sensing systems have been demonstrated for recognizing nucleic acid targets of three biomarkers in actual environmental samples, and these systems are summarized in Table 1.

4.1.1 Microorganisms

E. coli, *Staphylococcus aureus* (*S. aureus*), *Shigella flexneri* (*S. flexneri*), and *Listeria monocytogenes* (*L. monocytogenes*) are the most common causes of waterborne and foodborne diseases, so microorganism monitoring of water and food shows vital significance for controlling potential disease outbreaks [120]. Although PCR can detect these microorganisms, specific equipment and skilled people are necessary to analyze results, which limits POC diagnostics [121].

Liu et al. [122] established high-throughput RPA-CRISPR systems which detected ≥ 5 copies of pathogen DNA in 35 min. They successfully monitored *S. aureus* in 10 water samples from the mainstream and additional 7 samples from tributaries in the lower reaches of the Jialing River in Chongqing, China (Figure 3A). Subsequently, an amplification-free CRISPR-Cas12a-based magnetic relaxation switching (C-MRS) biosensor was developed to detect MRSA in food [123]. In 2024, Ma et al. [124] reported an efficient magnetic enrichment cascade single-step RPA-CRISPR-Cas12a assay for *S. aureus* detection with a lower LOD of 1 copy/ μ L. The single-step diagnostic system could prevent contamination and avoid false positives (Figure 3B). Besides detecting *S. aureus*, researchers developed CRISPR-Cas-based biosensors to detect *E. coli* O157:H7 in water and food [125], *Legionella pneumophila* (*L. pneumophila*) in environmental water [126], *Aeromonas hydrophila* (*A. hydrophila*) in human blood, stool and fish [127], *Pasteurella multocida* (*P. multocida*) in lung [128] and hygiene indicator bacteria in meat [129].

These examples prove that integrating CRISPR-Cas systems with various amplification and detection strategies can enable high-sensitivity monitoring of microorganisms, particularly in environmental water and food safety contexts. However, while some methods reduce or eliminate nucleic acid amplification, sample extraction remains complex especially for high-risk environmental samples like wastewater. In addition, the reliance on specialized reagents may pose challenges to the large-scale commercialization of these platforms for broader diagnostic use. Future efforts should focus on developing fully integrated single-pot CRISPR-Cas-based sensing systems with minimal and simple environmental sample preparation, making them more viable for commercial and field-deployable use.

4.1.2 Viruses

Similar to microorganisms, monitoring viruses in the environment is also essential to ensure public health. Cao et al. [114] first developed a portable paper device based on CRISPR-Cas12a and RT-LAMP for semiquantitative SARS-CoV-2 detection in wastewater (Figure 3C). Researchers find that the separate step of isothermal amplification may cause long-lasting amplicon aerosol contaminants,

resulting in false-positive results. To avoid aerosol contaminants, a one-tube RT-RPA-CRISPR-Cas13a-based assay has been proposed, detecting SARS-CoV-2 nucleic acids in wastewater with a LOD of 103 copies/mL (Figure 3D) [130]. To further simplify the test, Kang et al. [131] obtained viral RNA genomes via acid/base treatment without purification in the one-tube assay. Then, RT-RPA, CRISPR-Cas system activation, G-quadruplex (G4) cleavage, and G4-based colorimetric reaction were performed in a single tube with a LOD of 0.84 copies/ μ L. This facile colorimetric assay has been used to analyze contaminated surfaces and wastewater samples.

In addition, to reduce the interference of amplification, an amplification-free, interference-resistant, and portable single-vessel SARS-CoV-2 detection device was built by conjugating AuNPs with Raman reporting molecular and ssDNA probes. It detected SARS-CoV-2 as low as 200 copies/mL without preamplification within 45 min and was challenged for SARS-CoV-2 detection in simulated environmental and food samples [132]. Besides, there is also an electrochemiluminescence biosensor [133] and a Cas12a-facilitated portable plasmonic biosensor [134] for SARS-CoV-2 detection in food and sewage respectively. Except for the above samples, an RT-LAMP-CRISPR-Cas12a-based assay was developed to detect duck Tembusu virus (DTMUV) in 32 cloacal swabs and 22 tissue samples [135].

CRISPR-based biosensors for monitoring RNA viruses like SARS-CoV-2 in water and food offers both benefits and challenges. While some systems no longer require nucleic acid amplification, issues remain with sample extraction, especially for RNA viruses, whose instability demands specialized reagents or equipment. This complexity can limit accessibility, scalability, and precise quantification, particularly in low-resource settings. Thus, in addition to developing integrated single-pot CRISPR systems with simplified sample preparation, improving quantitative accuracy is crucial for more applications.

4.1.3 Antibiotic resistance genes

Wastewater is the main source of ARGs in the environment, leading to the dissemination of ARGs in urban water bodies, causing significant environmental and health concerns. To construct a method for on-site ARG assessment in wastewater, Mao et al. [117] combined CRISPR-Cas12a and LAMP for *ermB* detection. The biosensor possessed excellent sensitivity (with a LOD of 2.75×10^3 copies/ μ L) using fluorescence and later flow strips. A one-step approach for rapid detection of ARGs (*sulI*, *qnrA-I*, *mcr-I*, and *intI1*) was created by leveraging the trans-cleavage activity of Cas12a and RPA. Through monitoring these ARGs in 18 WWTPs in 9 cities across China during both peak and declining stages of the COVID-19 epidemic, they found an increased detection frequency of *mcr-I* and *qnrA-I* in wastewater during peak periods (Figure 3E) [136].

These two studies highlight the advancements in detecting antibiotic resistance genes (ARGs) in wastewater, focusing on CRISPR-based sensing systems and optimized monitoring approaches. As in the previous examples, sample preparation for environmental waters, such as wastewater, remains complex, which may limit ease of use in field applications. Additionally, CRISPR-Cas systems for ARG detection currently lack precision in quantitative measurements, and cost-effectiveness is a key consideration for broader application. Moreover, as ARGs are emerging contaminants, further research is needed to identify specific ARGs to prioritize as monitoring indicators and to establish threshold standards for maximum concentration levels in environmental monitoring.

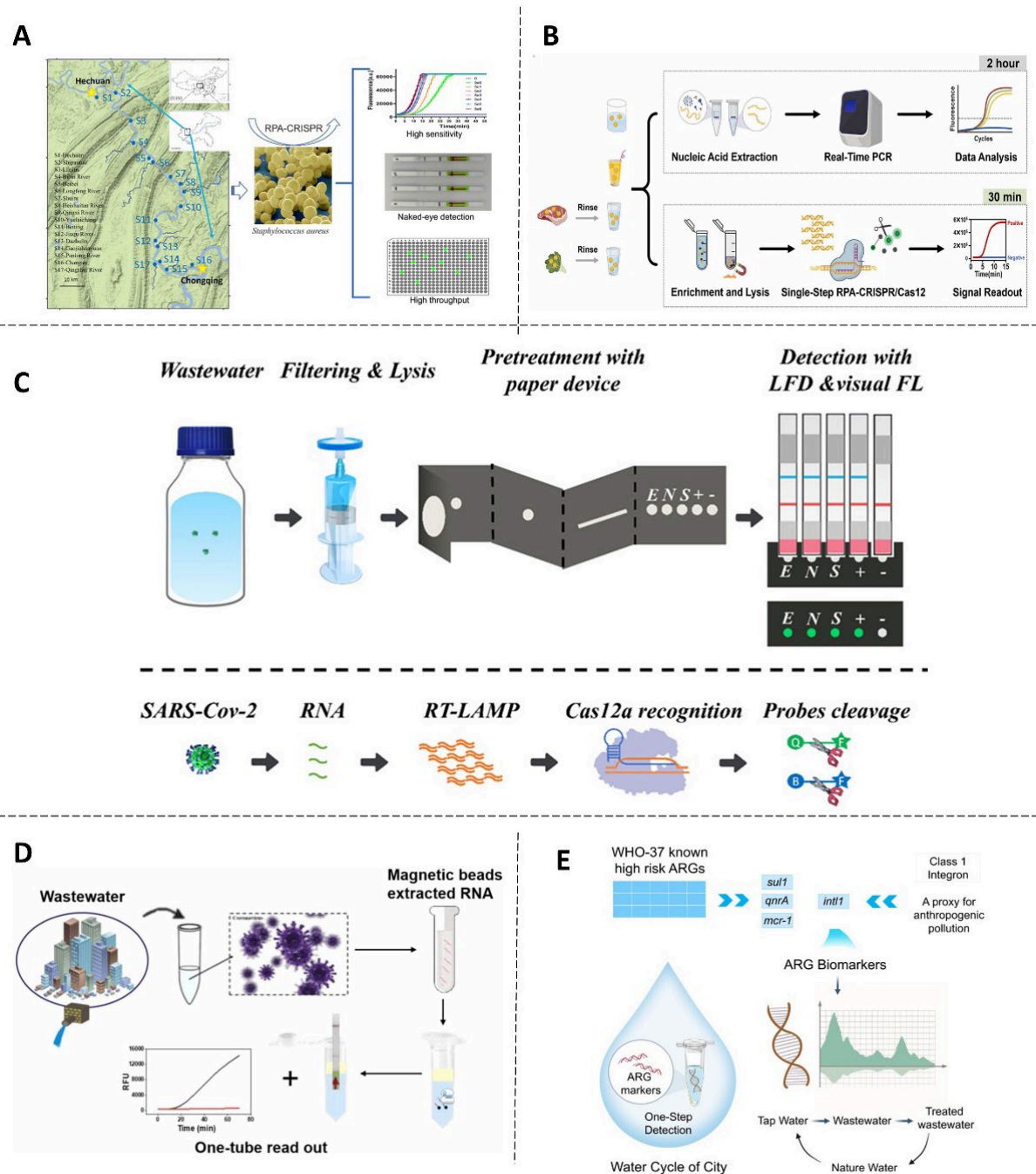


Figure 3. CRISPR-Cas-based systems to detect nucleic acid-based targets in real water samples. (A) Diagrams of CRISPR-Cas12a-mediated *S. aureus* detection in the natural water environment. Adapted with permission from Ref. [122]. (B) Single-step RPA-CRISPR-Cas12a assay for *S. aureus* detection in drinking water and food. Adapted with permission from Ref. [124]. (C) A portable RT-LAMP-CRISPR-Cas12a paper device to detect SARS-CoV-2 in wastewater. Adapted with permission from Ref. [114]. (D) A one-tube RT-RPA-CRISPR-Cas13 assay to detect wastewater. Adapted with permission from Ref. [130]. (E) A one-step RPA-CRISPR-Cas12a approach for monitoring ARGs in wastewater. Adapted with permission from Ref. [136].

Table 1 Application of sensors based on CRISPR-Cas systems for detecting nucleic acid targets in environmental samples.

Target	Cas	Amplification	LOD	Quantity	Time	Signal	Sample type	Refs.
Viruses								
SARS-CoV-2	Cas13a	RT-RPA	180 copies per reaction	Yes	40 min	Fluorescence	Wastewater	[130]
	Cas12a	RT-LAMP	N, E, and S genes-25, 310, and 10 copies/mL	Yes	1 h	Fluorescence/LFA	Wastewater	[114]
	Cas12a	/	2,300 copies/mL	Yes	30 min	Spectral Signal	Sewage water	[134]
	Cas12a	Entropy-driven amplification	32.80 aM	Yes	2 h	Electrochemiluminescence	Food bags, pork	[133]
	Cas12a	RT-RPA	0.84 copies/ μ L	Yes	30 min	Colorimetric	Contaminated surfaces, wastewater	[131]
	Cas12a	/	200 copies/mL	Yes	45 min	SERS Signal	Frozen shrimp, cold-chain food packaging	[132]
DTMUV	Cas12a	RT-LAMP	3 copies/ μ L	Yes	1.1 h	Fluorescence	Cloacal swabs, tissues	[135]
Microorganisms								
<i>S. aureus</i>	Cas12a	RPA	≥ 5 copies	Yes	35 min	Fluorescence/LFA	River water	[122]

	Cas12a	RPA	1 copy/ μ L	Yes	40 min	Fluorescence	Lake water, orange juice, pork, lettuce	[124]
<i>L. pneumophila</i>	Cas12a	RPA	3.8 copies/ μ L(F)/3800 copies/ μ L(C)	Yes	60 min/ 100 min	Fluorescence/Colorimetry	Simulated environmental water	[126]
<i>A. hydrophila</i>	Cas12a	dRAA	2 copies per reaction	Yes	45 min	Fluorescence	Stool and fish	[127]
MRSA	Cas12a	/	16 CFU/mL	Yes	1 h	Single Readout	Eggs, milk, pork	[123]
<i>P. multocida</i>	Cas12a	RPA	Single copy	Yes	1.5 h	Fluorescence	Multocida lung	[128]
<i>E. coli</i> O157:H7/ <i>S. aureus</i>	Cas12a	RPA	1 CFU/mL	Yes	50 min	Fluorescence	Milk	[125]
Hygiene indicator bacteria	Cas12a	PCR	1 cell/reaction	Yes	27 min	Fluorescence	Meat	[129]
ARGs								
<i>ermB</i>	Cas12a	LAMP	2.75×10^3 copies/ μ L	Yes	60 min	Fluorescence/LFA	Wastewater	[117]
<i>sulI</i> , <i>qnrA-I</i> , <i>mcr-I</i> , and <i>intI1</i>	Cas12a	RPA	1 aM for <i>sulI</i> , <i>mcr-I</i> , and <i>intI1</i> , 10 aM for <i>qnrA-I</i>	Yes	20 min	Fluorescence	Wastewater	[136]

Table 2 Application of sensors based on CRISPR-Cas systems for detecting non-nucleic acid targets in water samples.

Target	LOD	Quantity	Time	Signal	Sample type	Refs.
Heavy metal ions						
Pb ²⁺	0.48 nM	No	60 min	Fluorescence	Drinking water	[138]
	86 fM	No	30 min	Fluorescence	Soil	[139]
	1 pg/L	Yes	40 min	Fluorescence	Lake water	[140]
Cd ²⁺	1.25 pM	Yes	20 min	Fluorescence	Tap water, lake water, pond water, river water	[141]
	60 pM	No	60 min	Fluorescence	Lake water	[142]
Hg ²⁺	40 fM	Yes	30 min	Electric	Tap water, river water, sewage	[143]
	23 pM	No	30 min	Fluorescence	River water	[144]
Antibiotics						
AMP	2.06 nM	Yes	45 min	Fluorescence	River water	[145]
	10 fM	Yes	50 min	Electric	Wastewater	[149]
TOB	0.83 nM	Yes	90 min	Electric	Tap water, pool water, spring water, river water	[147]
	3.719 pM	Yes	60 min	Fluorescence	Lake water	[146]
KAN	60 fM	Yes	50 min	Electric	Wastewater	[149]

	1 pM	Yes	90 min	Electric	River water	[148]
SDM	2.86 pg/mL	Yes	50 min	Fluorescence	Lake water	[150]
Organic pollutants						
DEHP	0.02 pM	Yes	50 min	Colorimetry	River water	[154]
BaP	4.971 fg/mL	Yes	/	Fluorescence	Lake water	[157]
AFB1	2.57 ag/mL	Yes	/	Fluorescence	Lake water	[157]
CAP	794.6 fg/mL	Yes	/	Fluorescence	Lake water	[157]
PCBs	128 fg/mL	No	55 min	Fluorescence	River water	[155]
E2	0.08 nM	Yes	20 min	Fluorescence	Sewage	[156]
BPA	0.06 nM	Yes	20 min	Fluorescence	Sewage	[156]
OPs	0.135 ng/mL	No	40 min	Fluorescence	Lake water	[158]
Others						
MCs	19 pg/mL	Yes	210 min	Fluorescence	River water	[160]
	3.02 pg/mL	No	60 min	Fluorescence	River water	[161]
	4.53 pg/mL	Yes	30 min	Colorimetry	Tap water, river water	[162]

C. parvum oocyst

Single oocyst

Yes

90 min

Fluorescence

Water

[163]

4.2 Non-nucleic acid-based chemical and biological detection

Although CRISPR-enabled sensors are mainly focused on nucleic acid detection, recent advancements have shown that combining this innovative mechanism with DNAzymes, aptamers and antigen-antibody reactions can achieve susceptible detection of non-nucleic acid targets such as heavy metals, antibiotics, organic pollutants, and certain toxins in the environment (Table 2).

4.2.1 Heavy metals

Heavy metal water contamination is a global health problem. In the most severely affected resource-limited areas, the lack of skilled personnel and medical facilities has resulted in populations inadvertently consuming water contaminated with heavy metals [137]. Since 2021, CRISPR-Cas12a has been reported as an effective signal amplification strategy for detecting the concentration of heavy metal ions, such as lead (Pb^{2+}), cadmium (Cd^{2+}), and mercury (Hg^{2+}) in environmental samples. The key to these sensors resides in their ability to selectively identify heavy metal ions and subsequently activate CRISPR-Cas12a systems.

The detection of Pb^{2+} typically relies on DNAzyme probes. Chen et al. were the first to combine CRISPR-Cas12a system with DNAzyme probes to detect Pb^{2+} in drinking water [138]. DNAzymes identify Pb^{2+} and convert the chemical signal into readable biomolecular signals, thereby activating the CRISPR-Cas12a trans-cleavage effect. The system could detect Pb^{2+} at concentrations as low as 0.48 nM and used a portable 3D-printed device for on-site detection (Figure 4CA). Subsequently, two studies improved the design with a spherical nucleic acid (SNA) fluorescence probe, using AuNPs-DNAzyme with excellent nuclease resistance [139, 140]. Liang et al. also utilized a Cd^{2+} -specific DNAzyme to detect Cd^{2+} in environmental water [141]. Another SDA aptasensor for Cd^{2+} detection achieved a LOD of 60 pM in real water samples [142]. The third type of sensor was based on the coordination chemistry of thymine- Hg^{2+} -thymine (T- Hg^{2+} -T) structures, primarily designed for Hg^{2+} detection. This technology avoids the use of expensive chemically modified nucleic acid probes [143, 144].

These CRISPR-based heavy metal detection systems demonstrate that combining DNAzymes, HCR, and strand displacement amplification with CRISPR can greatly improve their accessibility and usability for field detection [138]. Adjusting DNAzyme types or adding specific probes also makes these systems versatile for detecting multiple metals. However, challenges like complex sample preparation, limited quantitative precision, and high costs may limit their scalability, especially in low-resource settings. Future research should aim to simplify sample processing, enhance accuracy, reduce costs, and enable multi-metal detection to expand their use in environmental monitoring.

4.2.2 Antibiotics

The extensive use of antibiotics in medical treatment and livestock farming results in widespread residue in the environment. Current research primarily focuses on utilizing aptamers to recognize antibiotics (Figure 4B), enabling sensors to convert this information into chemical signals. Hu et al. [145] first proposed the combination of element probe with CRISPR-Cas14 biosensing in 2021. The design includes a locking strand with two ampicillin (AMP) aptamer sequences, which bind to the activator strand. Upon AMP recognition, the aptamer releases the activator strand to bind with CRISPR-Cas14, activating its trans-cleavage activity and cleaving the probe. The cleaved ssDNA is captured and removed by streptavidin-coated MBs.

Subsequent studies combined different aptamer recognition with CRISPR-Cas12a systems to develop biosensors for detecting tobramycin (TOB) [146, 147], kanamycin (KAN) [148], AMP [149], and sulfadimethoxine (SDM) [150] in water samples. Among them, one study compared two biosensors based on CRISPR-Cas12a for detecting TOB [146]. These biosensors utilized ssDNA and dsDNA to trigger CRISPR-Cas12a collateral cleavage activity. They found that sensors utilizing double-stranded activators exhibited unique advantages in sensitivity, linear range, and practical feasibility. The recent study designed a sensitive and versatile electrochemical biosensor based on the HCR triggering CRISPR-Cas12a system, utilizing NH₂-Cu-MOF as the signal molecule. This biosensor successfully detected KAN and AMP in wastewater, with detection limits of 60 fM and 10 fM, respectively [149].

CRISPR-enabled sensors for antibiotic detection utilizing element probes and DNA activators, offer high sensitivity and flexibility, allowing for trace-level detection of antibiotics in water environments. The integration of portable readout devices (such as glucometers and electrochemical sensors) further enhances their feasibility for on-site monitoring in real-world conditions. Their ability to detect non-nucleic acid substances like antibiotics extends the application of CRISPR-based biosensors. Although these systems exhibit strong sensitivity and portability, further research is needed to improve their stability, accuracy, and cost-effectiveness for commercialization.

4.2.3 Organic pollutants

Human activities and industrial production lead to an increasing release of organic pollutants into the environment. For example, persistent organic pollutants such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and organophosphorus pesticides (OPs) may accumulate in organisms and pose a threat to human health [151-153]. There is a great need for rapid, convenient, and on-site detection of organic pollutants, but CRISPR-Cas-based detection is still in the initial stage.

The biggest challenge of CRISPR-enabled sensors is to find a suitable sensing receptor. Aptamers are the most widely used sensing receptors for detecting organic pollutants (Figure 4C), which were used to detect di(2-ethylhexyl) phthalate (DEHP) [154], 2,3',5,5'-tetrachlorobiphenyl (PCB72) and 3,3',4,4'-tetrachlorobiphenyl (PCB77) [155], 17 β -estradiol (E2) and bisphenol A (BPA) [156]. In these sensors, adjusting aptamer to activator DNA ratio can reduce signal leakage and delay the release of activator DNA. Additionally, sensitivity and LOD can be optimized by trimming the sequence of aptamer. Another study developed a CRISPR-Cas12a-driven intelligent POCT platform for detecting aflatoxin B1 (AFB1), benzo[a]pyrene (BaP), and capsaicin (CAP) in lake water, which utilized antibody/antigen interactions to induce fluorescent signals [157]. A novel OP sensor based on acetylcholinesterase (AChE) and MnO₂ nanosheets mediated CRISPR-Cas12a reaction. In the presence of target OPs, the hydrolysis of ATCh by AChE was inhibited, resulting in a change in fluorescence signal, enabling effective quantification of OPs [158].

These studies suggest that CRISPR-enabled sensors combined with signal amplification techniques (such as catalytic hairpin assembly and DNA aptamers) can detect trace levels of persistent organic pollutants (POPs) in environmental waters, which is essential for environmental monitoring. However, as with the previously discussed CRISPR-Cas systems for detecting heavy metals and antibiotics, there remains room for improvement in areas such as sample preparation, quantitative accuracy, equipment dependency, multi-pollutant detection capability, cost-effectiveness, and long-term stability.

4.2.4 Other contaminants

CRISPR-Cas systems have also been used to detect toxins in water samples, such as microcystins (MCs) released from cyanobacteria and gliotoxin isolated from fungi. Benefiting from the successful selection of a high-affinity DNA aptamer by Zourob et al. [159], several studies have combined aptamer recognition with the CRISPR-Cas systems to detect MC-LR. For instance, a CRISPR-Cas14a system was activated by complementary DNA released from the MC-LR aptamer, followed by Cas14a-catalyzed nucleic acid cleavage reactions that generated fluorescent signals [160]. In another two studies, CRISPR-Cas12a was employed as a signal amplification tool where the release of cDNA from the MC-LR aptamer activated CRISPR-Cas12a for on-site detection [161, 162]. Another noteworthy study used the ultra-sensitive CRISPR-Cas system to detect waterborne parasites (Figure 4D). This sensor integrated antibody-based recognition with CRISPR-Cas12a-mediated fluorescence signal amplification via an antibody-DNA conjugate. It demonstrated sensitive detection of *Cryptosporidium parvum* (*C. parvum*) oocysts, reducing the dependence on microscopy for detection [163].

CRISPR-based sensors demonstrate the advantages of CRISPR in sensitivity, specificity, and field use, especially when combined with techniques like aptamers, dual-amplification systems, and plate readers. However, many CRISPR methods involve multiple components or amplification steps, adding complexity and cost, which can limit scalability in low-resource settings.

In summary, to improve the on-site use of CRISPR-based sensors for non-nucleic-acid targets in water, future efforts should focus on simplifying equipment, reducing reliance on specialized reagents, enhancing stability in varied conditions, expanding multi-target capabilities, and creating commercially viable platforms.

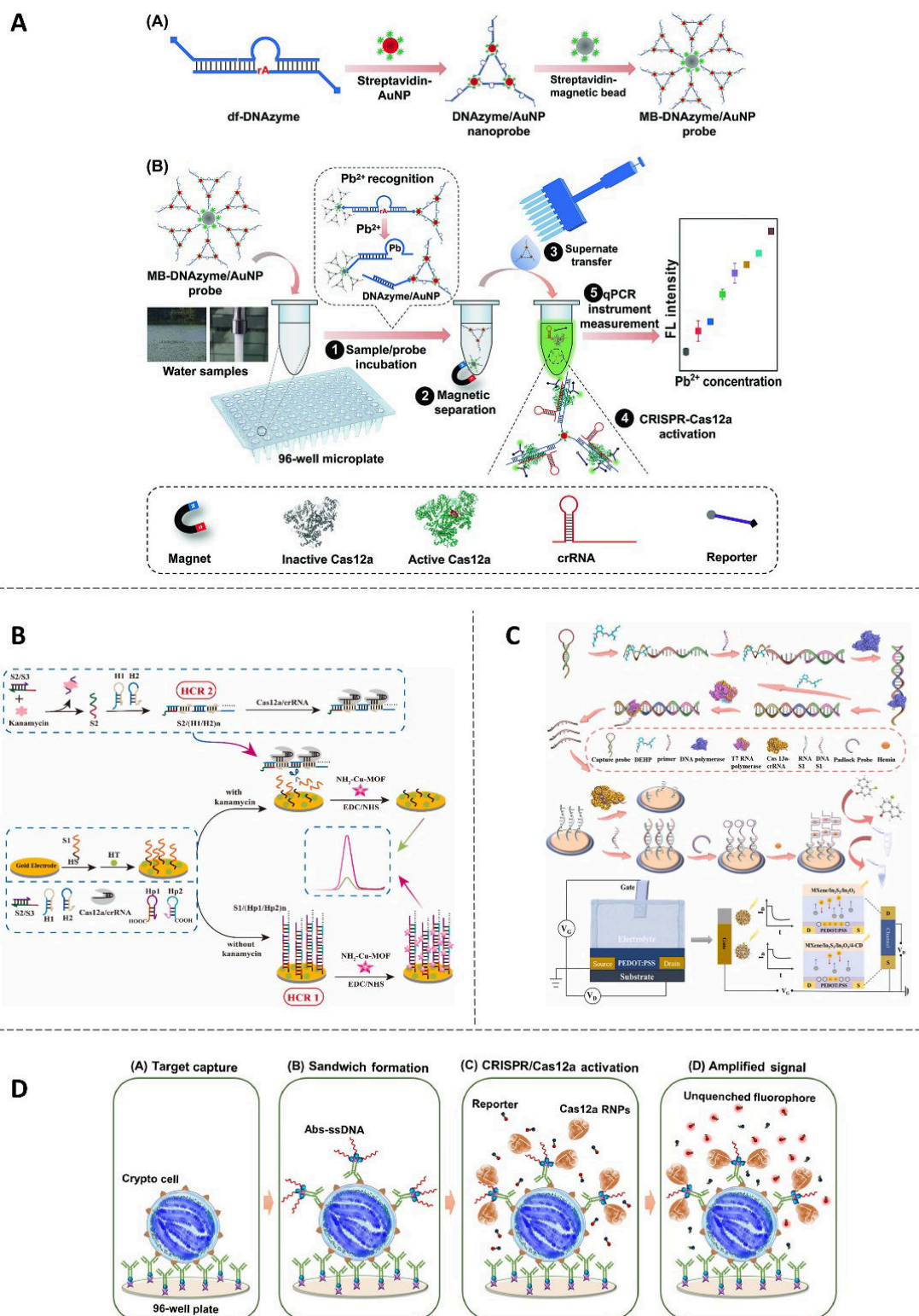


Figure 4. CRISPR-Cas-based systems to detect non-nucleic acid-based targets in real water samples. (A) Schematics for dual-functional DNzyme powered CRISPR-Cas12a sensor for of Pb^{2+} detection in freshwater. Adapted with permission from Ref. [140]. (B) Illustration of the electrochemical sensing platform based on HCR and CRISPR-Cas12a cascade reaction circuit for antibiotic detection. Adapted with permission from Ref. [149]. (C) Illustration of colorimetry-assisted organic photoelectrochemical transistor sensing detection mechanism based on CRISPR-Cas13a. Adapted with permission from Ref. [154]. (D) Schematics for the CRISPR-Cas12a involved whole cell biosensing system for detection. Adapted with permission from Ref. [163].

5. Conclusions and future perspectives

As public awareness of the impact of environmental contaminants on human health intensifies, there is a growing demand for rapid, cost-effective, and equipment-free methods to identify contaminants and monitor biomarkers in the environment. The integration of CRISPR-Cas technologies with nucleic acid amplification strategy shows the potential to develop a new sensing strategy, including biosensors and chemical sensors with improved specificity and sensitivity [164]. To enable the rapid and on-site monitoring of new contaminants, researchers have started focusing on building portable miniaturized CRISPR-Cas-based biosensing platforms using engineering approaches. Nowadays, CRISPR-Cas systems can detect different contaminants like viruses, heavy metal ions and organic pollutants in the environment. It is worth noting that wastewater surveillance based on CRISPR-Cas technology provides insights into population lifestyle, health status, and toxicant exposure. The consistent monitoring of biomarkers within sewage systems also elucidates spatial variations and temporal fluctuations in biomarker concentrations, which is instrumental in the early diagnosis and prevention of disease outbreaks [165].

It is worth noting that CRISPR-enabled sensors offer distinguished strengths in sensitivity, specificity, and adaptability, making them highly suitable for environmental contaminants detection. When integrated with amplification and microfluidic techniques, these sensors can achieve trace-level detection, which is indispensable for identifying contaminants in the environment. Additionally, CRISPR-enabled sensors demonstrate versatility and flexibility in detecting various targets beyond nucleic acids, such as heavy metals, antibiotics and other organic pollutants, by employing aptamers, DNAzymes, and element probes [139, 140]. The use of portable readout devices, like glucometers or electrochemical sensors, further enhances their feasibility for on-site monitoring. These innovations enable CRISPR-Cas systems to respond to a broad spectrum of environmental contaminants.

However, there remain some challenges in CRISPR-Cas-based systems for rapid monitoring of environmental contaminants. Initially, the majority of CRISPR-Cas-based systems require integration with auxiliary technologies, which can potentially lead to issues such as non-specific amplification and cross-contamination. For example, the necessity of a two-step process can be cumbersome for users, particularly in field detection scenarios, which can be time-consuming and may reduce accuracy due to risks of aerosol contamination or cross-interference. Additionally, when analyzing environmental samples such as soil, water, and food, the prerequisite of DNA/RNA extraction from these matrices can be inconvenient and may necessitate handling large sample volumes, which can be impractical and limit the participation of individuals without specialized training. Moreover, most CRISPR-based methods are currently unable to perform direct field quantification and often require standardization via PCR techniques. Finally, environmental stability and reliability under diverse field conditions are essential but can be compromised due to the specific temperature and handling requirements of CRISPR reactions.

Future advancements could prioritize developing fully integrated field-deployable platforms that require minimal sample preparation and eliminate the need for pre-amplification to enhance the applicability of CRISPR-Cas systems. Designing temperature-independent CRISPR reactions would further improve accuracy, save time, and reduce the risk of contamination. Additionally, modifying Cas enzymes to achieve cleavage-based strategies targeting each gene uniquely or designing cleavage-specific probes could enable simultaneous monitoring of multiple targets in diverse sample types. In the future, the convergence of CRISPR-Cas systems with complementary technologies is anticipated to yield portable, miniaturized, and commercialized biosensors. In addition, more ideas can be broadly discussed, such as

integration with artificial intelligence (AI) technology and machine learning [166, 167]. For example, sensitivity and precision of CRISPR-enabled sensors are critical in facilitating the acquisition of extensive data. By embedding AI analytical systems into real-time monitoring sensors, AI can swiftly assess whether contaminants detected in the environment might pose risks to local ecosystems and populations. AI-driven data analysis coupled with industrial-scale production to reduce costs, could pave the way for the commercial development of compact devices. These advanced devices are promised to facilitate precise diagnostic assessments, including the monitoring of specific biomarkers and the detection of various environmental contaminants.

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