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Review

Nucleic Acid Detection Using CRISPR/Cas Biosensing Technologies

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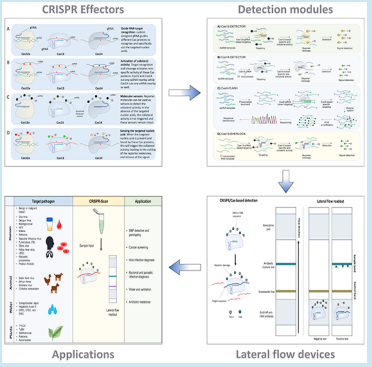
ABSTRACT: For infectious diseases, rapid and accurate identification of the pathogen is critical for effective management and treatment, but diagnosis remains challenging, particularly in resource-limited areas. Methods that accurately detect pathogen nucleic acids can provide robust, accurate, rapid, and ultrasensitive technologies for point-of-care diagnosis of pathogens, and thus yield information that is invaluable for disease management and treatment. Several technologies, mostly PCR-based, have been employed for pathogen detection; however, these require expensive reagents and equipment, and skilled personnel. CRISPR/Cas systems have been used for genome editing, based on their ability to accurately recognize and cleave specific DNA and RNA sequences. Moreover, following recognition of the target sequence, certain CRISPR/Cas systems including orthologues of Cas13, Cas12a, and Cas14 exhibit collateral nonspecific catalytic activities that can be employed for nucleic acid detection, for example by degradation of a labeled nucleic acid to produce a fluorescent signal. CRISPR/Cas systems are amenable to multiplexing, thereby enabling a single diagnostic test to identify multiple targets down to attomolar (10^{-18} mol/L) concentrations of target molecules. Developing devices that couple CRISPR/Cas with lateral flow systems may allow inexpensive, accurate, highly sensitive, in-field deployable diagnostics. These sensors have myriad applications, from human health to agriculture. In this review, we discuss the recent advances in the field of CRISPR-based biosensing technologies and highlight insights of their potential use in a myriad of applications.



KEYWORDS: *CRISPR-Cas, biosensing platforms, nucleic acid detection, diagnosis*

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Infectious diseases account for roughly a quarter of human deaths globally,[1] and when it comes to the distribution of mortality rates in low income and low-middle income countries, this proportion is significantly higher. Rapid, accurate, cost-effective detection and identification of pathogens is key for the control and management of infectious disease.[2] However, key innovations are needed to develop affordable point-of-care diagnostics that can reach populations in under-resourced areas lacking healthcare infrastructure. Diagnostics that require processing of samples in a well-equipped centralized laboratory slow diagnosis in resource-limited settings, where a lack of infrastructure and skilled medical personnel preclude effective diagnosis. Therefore, the development of reliable and rapid diagnostic tests that can be conducted outside the clinical laboratory is vital for effective disease treatment and management. For example, affordable, sensitive, and specific diagnostic products amenable to deployment at point-of-care settings for only four infectious diseases (malaria, bacterial pneumonia, tuberculosis, and syphilis) could save an estimated 1.2 million lives worldwide each year.[3,4] Development of rapid initial screening tests that can be used at the point-of-care will minimize the delay and expense of testing in centralized laboratories.[2]

Accurate identification of the causal pathogen requires pathogen-specific biomarkers, which can be antigens or nucleic acid sequences. Therefore, current diagnostics rely heavily on culturing, biomarker analysis via polymerase chain reaction (PCR) and antibody-based methods, and even genome sequencing.[5−10] However, these methods require sophisticated infrastructure and skilled technical staff; these requirements limit their deployment in developing countries and in disease outbreaks.[11] Moreover, development of many assays, particularly antigen-based assays, requires a long lead time for generation of antibodies; this provides another disadvantage for emerging or rapidly evolving pathogens. Emerging innovative diagnostics platforms promise to overcome these limitations and offer inexpensive, fast, sensitive, specific, and simple diagnostics.[12] The World Health Organization has developed the ASSURED criteria (affordable, sensitive, specific, user friendly, rapid and robust, equipment-free and deliverable to end users) to ensure that the products can work in under-resourced areas or where disease outbreaks occur.[13,14]

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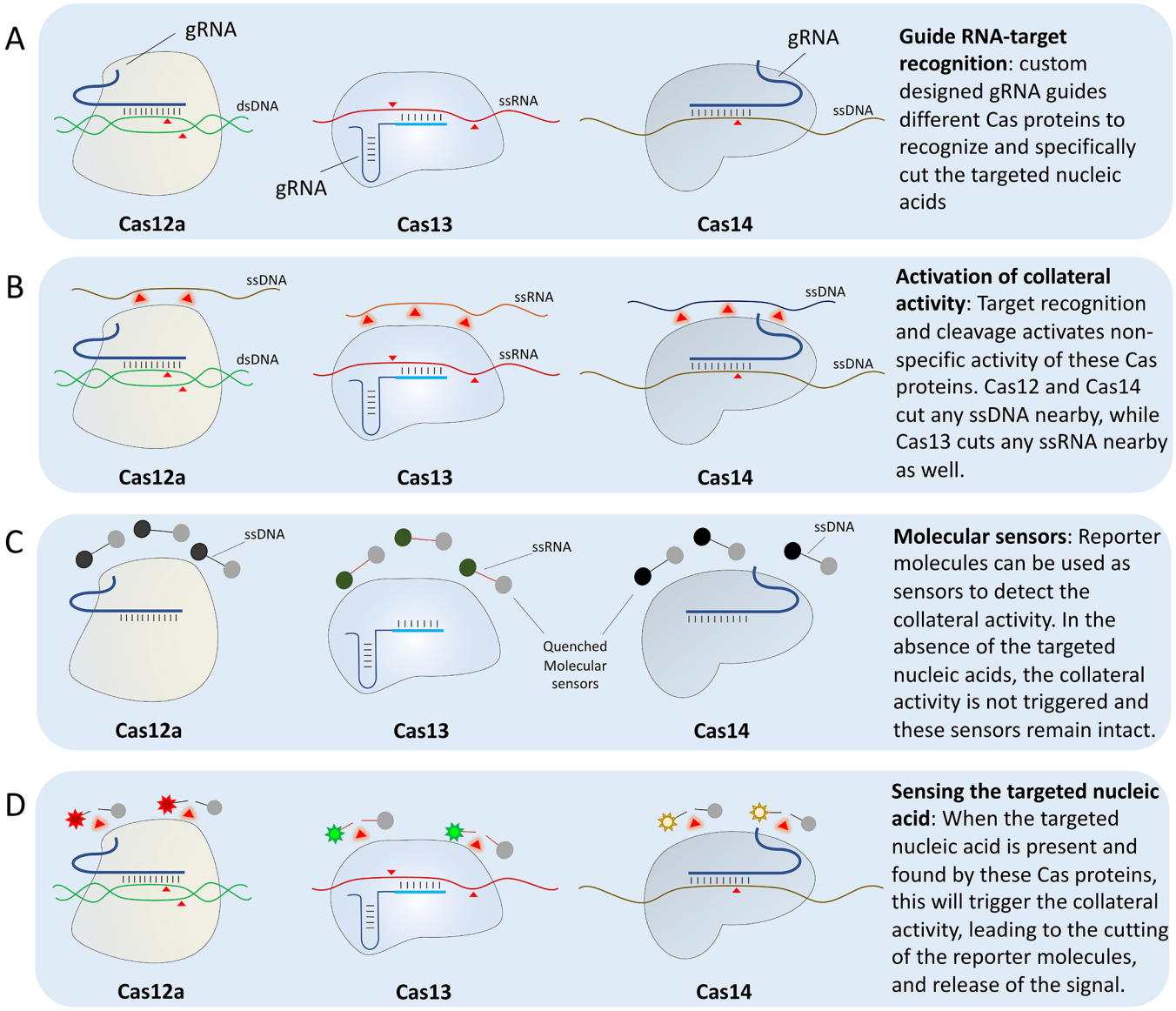

Figure 1. Overview of CRISPR-Cas enzymes activities and their catalytic mechanisms for nucleic acid detection. (A) Cas12, Cas13, and Cas14 enzymes are single effector proteins that can be guided by a single guide RNA (gRNA) to bind its targeted sequence. Cas12 and Cas14 target dsDNA and ssDNA, respectively, and Cas13 targets solely ssRNA substrates. (B) A common characteristic of these enzymes is the activation of nonspecific, collateral activity, upon target recognition. (C) The high specificity and collateral activity of these enzymes can be harnessed as CRISPR-based molecular sensors. Reporter molecules composed of a nucleic acid sequence that is a substrate for the *in trans* collateral activity of Cas enzymes and labeled with a fluorophore at one end and a quencher at the other end are used as sensing molecules. (D) When these Cas enzymes recognize and bind to their target nucleic acid, their collateral activities are triggered, leading to the degradation of the reporter sequence and thus the emission of the fluorescent molecule. Such molecular sensors can be developed as ultrasensitive methods for detection of nucleic acids of interest.

CRISPR systems can be employed for sensitive and specific detection of pathogen-specific nucleic acids, thereby unlocking the potential for diverse in-field diagnostic and genotyping applications. For example, CRISPR-based biosensing platforms on paper substrates are poised to revolutionize diagnosis of pathogens. Other CRISPR-Cas systems have been developed for detection of nucleic acids and biomarkers from pathogens, promising the development of low-cost, sensitive, specific diagnostics for infectious diseases.[15] In this review, we highlight the recent developments in using CRISPR/Cas systems to build biosensing platforms. We provide examples of harnessing CRISPR/Cas biosensing technologies to detect, diagnose, and genotype various pathogenic and nonpathogenic nucleic acids. In addition, we provide insights into potential applications using these CRISPR-based diagnostic platforms for inexpensive, affordable, and field-deployable point-of-care diagnosis.

HARNESSING CRISPR/CAS SYSTEMS FOR BIOSENSING APPLICATIONS

CRISPR (clustered regularly interspaced short palindromic repeats) was first discovered in the 1980s and has become the tool of choice for genome editing.[16,17] Up to date, all identified CRISPR/Cas systems are classified into two main classes that are further subdivided into different types and subtypes based on the organization of their loci and signature proteins. Class I CRISPR/Cas systems include type I, III, and IV, which employ multisubunit effector complexes. On the other hand, Class II CRISPR/Cas systems use single, RNA-guided, multidomain

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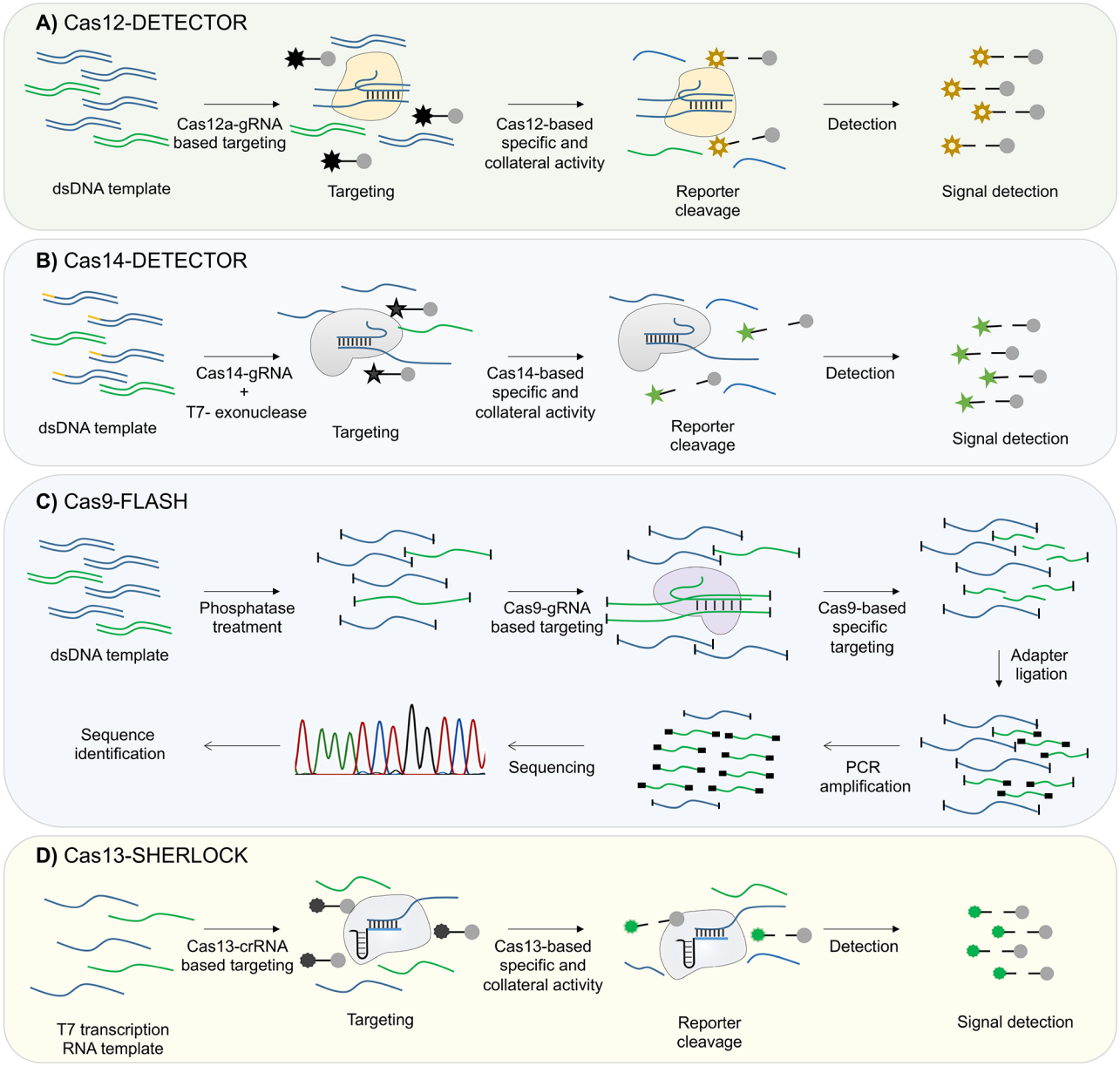

Figure 2. Schematic of the steps of different CRISPR-Cas detection approaches. (A) Cas12-based nucleic acid detection. The RPA (recombinase polymerase amplification) amplified DNA templates are used directly as targets for the Cas12-target specific gRNA complex. Upon target recognition and cleavage, the Cas12 collateral activity results in the degradation of the fluorescent reporter, which can be subsequently detected indicating the presence of the target. (B) Cas14-based nucleic acid detection. Cas14 can recognize and bind efficiently to ssDNA substrates. Targeted ssDNA is amplified by RPA using modified primers that will modify one end of the generated dsDNA products (yellow end). The strand with modified end will be resistance to T7 exonuclease treatment that will degrade the unmodified DNA strand, leaving the modified strand as a substrate for Cas14-gRNA complex. Upon target recognition and cleavage, the Cas14 collateral activity will results in the degradation of the fluorescent reporter, which can be subsequently detected indicating the presence of the target. (C) Cas9-based nucleic acid detection. A pool of dsDNA fragments treated with phosphatase is subjected to Cas9 mediated specific cleavage of the target sequence. In contrast to the uncleaved product, the cleaved DNA ends are then ligated to adapters. Adapter-specific PCR primers are used to amplify the cleaved product, followed by sequencing to identify the target sequence. (D) Cas13-based nucleic acid detection. Following the reverse transcription of the RNA substrate, the RPA-amplified DNA templates are *in vitro* transcribed using T7 *in vitro* transcription to generate RNA templates that are recognized by Cas13-gRNA complexes. Upon target recognition and cleavage, Cas13 collateral activity results in the degradation of the fluorescent reporter, which can be subsequently detected indicating the presence of the target.

Cas proteins to recognize and cleave target sequences. Class II CRISPR/Cas systems encompass multiple types, including type II systems such as Cas9, type V, including subtypes Cas12 and Cas14 (designated now as Cas12f), and type VI, including Cas13 systems.[18]

After recognition of the target sequence (guided by a single guide RNA, sgRNA), a CRISPR-associated nuclease (Cas) cleaves the target DNA, creating a site-specific DNA double-strand break (DSB). The structure of the sgRNA scaffold depends on the Cas protein used. CRISPR/Cas9 is most widely used for genome engineering applications.[19] However, different Cas enzymes have different activities that can be advantageous for diagnostic applications. CRISPR/Cas12a produces staggered-end DSBs, CRISPR/Cas13 targets single-stranded RNA

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(ssRNA), and CRISPR/Cas14 targets single-stranded DNA (ssDNA).[20−22] Interestingly, following recognition and cleavage of the specific target, Cas12a, Cas13, and Cas14 exhibit collateral, nonspecific activities against ssDNA or ssRNA (in the case of Cas13);[20,22−24] these activities can be utilized for nucleic acid detection applications. For example, a ssRNA or ssDNA reporter can be cleaved by Cas13, Cas12a, or Cas14 resulting in a fluorescent signal as a readout, produced by activation of molecular sensors (Figure 1).[20,25,26] In addition, the specific cleavage activity of Cas9 has been used to develop a highly sensitive DNA biosensing platform.[27]

A. Biosensing Platforms Based on DNA-Targeting CRISPR/Cas Systems. CRISPR-Cas12a (Cpf1) effector proteins are RNA-guided enzymes that cut DNA as components of bacterial adaptive immune systems. Similar to CRISPR-Cas9, Cas12a has been used for genome editing on the basis of its ability to generate targeted DSBs.[23] Interestingly, the binding of Cas12, specifically LbCas12a, to its target DNA unleashes nonspecific ssDNA cleavage activity, which completely degrades other existing ssDNA molecules. This target-activated, *in trans* ssDNA collateral activity of Cas12 has been used for molecular diagnostics of DNA molecules, and led to the development of the DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter) method (Figure 2A). DETECTR has shown great ability for viral detection from clinical specimens at high detection sensitivity.[23] For example, the collateral activity of Cas12 was used as a diagnostic platform to detect *Mycobacterium tuberculosis* in clinical samples.[28] Other studies have improved the utility of Cas12 for rapid detection of nucleic acid at the subattomolar level.[29,30] For example, HOLMES (a one-hour low-cost multipurpose highly efficient system) was developed for fast detection of target DNA as well as RNA.[30] Intriguingly, HOLMES was further improved (HOLMESv2) to allow more than just simple nucleic acid detection. For example, HOLMESv2 can specifically discriminate single nucleotide polymorphisms (SNPs), conveniently quantify target nucleic acids with a one-step system combined with LAMP amplification in a constant temperature, and accurately quantify target DNA methylation with the combination of Cas12b detection and bisulfite treatment.[31] Besides its ability for nucleic acid detection, Cas12 has been developed as high throughput platform termed CaT-SMelor (CRISPR-Cas12a- and aTF-mediated small molecule detector) for detection of small molecules. The principle of CaT-SMelor to detect small molecules still relies on the idea of triggering Cas12 collateral activity upon target recognition to cleave a fluorophore quencher (QC)-labeled ssDNA probe molecule and releasing a detectable signal. However, here, the Cas12a-activating DNA contains a motif that is recognized by the CBD (cellulose binding domain)-bound aTF (allosteric transcription factor), which is in turn immobilized on a MC (microcrystalline cellulose). Binding of DNA to aTF sequesters it from recognition by Cas12. However, in the presence of certain, sought-after, small molecules, a conformational change of aTF occurs, resulting in the release of the DNA that triggers Cas12 ssDNA trans cleavage collateral activity. CaT-SMelor has been used to detect various small molecules, including uric acid and *p*-hydroxybenzoic acid in clinical human blood samples with high sensitivity.[32]

Recently, a novel class 2 type V CRISPR/Cas system was discovered, the CRISPR/Cas14. This newly discovered Cas14 possesses a specific targeting activity against single stranded DNA substrates in a PAM-independent manner.[20] Like Cas12, Cas14 harbors a RuvC domain, a characteristic of Class II type V Cas enzymes. Interestingly, the Cas14 *in cis* cleavage of targeted ssDNA leads to indiscriminate cleavage of ssDNA *in trans*, like Cas13 and Cas12 do for RNA and dsDNA, respectively.[20] The lack of requirement for a PAM sequence, its ability to recognize ssDNA targets, and its small size, render Cas14 as another excellent candidate for ssDNA and dsDNA detection purposes. The target-dependent, nonspecific DNase activity of Cas14 was utilized as a DNA detection platform for a high-fidelity detection system (Cas14-DETECTR) (Figure 2B).[20,33] Interestingly, unlike Cas12-DETECTR, Cas14-DETECTR has shown stronger specificity and activity which allows high-fidelity detection of DNA SNPs.[20] The development of Cas14-DETECTR as a highly sensitive CRISPR-based detection method allows the diagnosis of important ssDNA pathogens as well as a high-fidelity and robust genotyping tool for the detection of SNPs without the constraint of the presence of a PAM sequence.

The impressive advancement in this field has led scientists to creatively exploit existing CRISPR/Cas technologies (that do not exhibit collateral activity upon target recognition) as highly sensitive diagnostic platforms. Recently, Quan et al. utilized the high specificity and activity of Cas9 to develop FLASH (finding low abundance sequences by hybridization). This next generation CRISPR/Cas9-based diagnostic method takes advantage of Cas9 specificity and efficiency to enrich sequences of a specific target.[27] The sample DNA is blocked by phosphatase treatment and subsequently treated with Cas9 guided by a single guide RNA specific to the sequence of interest. The ends of the cleavage products will be amenable for ligation of universal sequencing adapters. By using the adapters for specific amplification, the target sequences will be enriched over background and will be ready for subsequent sequencing enabling high multiplexing, precision and identification of the target sequence (Figure 2C). The ability of FLASH to reveal the sequence identity of the target DNA is an advantage over the other collateral-based diagnostic platforms. This system allowed the detection of antimicrobial resistance genes in different clinical samples including respiratory fluid and dried blood samples.[27]

B. Biosensing Platforms Based on RNA-Targeting CRISPR/Cas System. Class II type VI CRISPR/Cas13 effector proteins contain a single RNA guided ribonuclease that specifically targets single stranded RNAs (ssRNAs) to protect bacteria from invading genetic elements.[34] All Cas13 proteins studied to date have dual enzymatic nuclease activity that are crucial for optimal RNA interference. One activity is responsible for pre-crRNA processing to generate mature crRNAs that guide Cas13 to its target. The other activity mediated by the two conserved Cas13 signature high eukaryotic and prokaryotic nucleotide-binding domains (HEPN) is responsible for the specific cleavage of the targeted RNA.[35] Biochemical and functional studies have allowed the rapid development of this system for a variety of exciting RNA targeting and manipulation applications in different systems.[36−40] Interestingly, upon activation of Cas13 enzyme by binding and specific cleavage of its target RNA, a nonspecific, trans-ssRNA cleavage is triggered, leading to collateral degradation of other ssRNAs in its vicinity.

A study by East-Seletsky et al. demonstrated the potential utility of this collateral activity to sense and detect the presence of specific transcripts in a sample. The specificity and target RNA binding-based collateral cleavage activity of Cas13 was used to detect the presence of specific nucleic acid in a pool of

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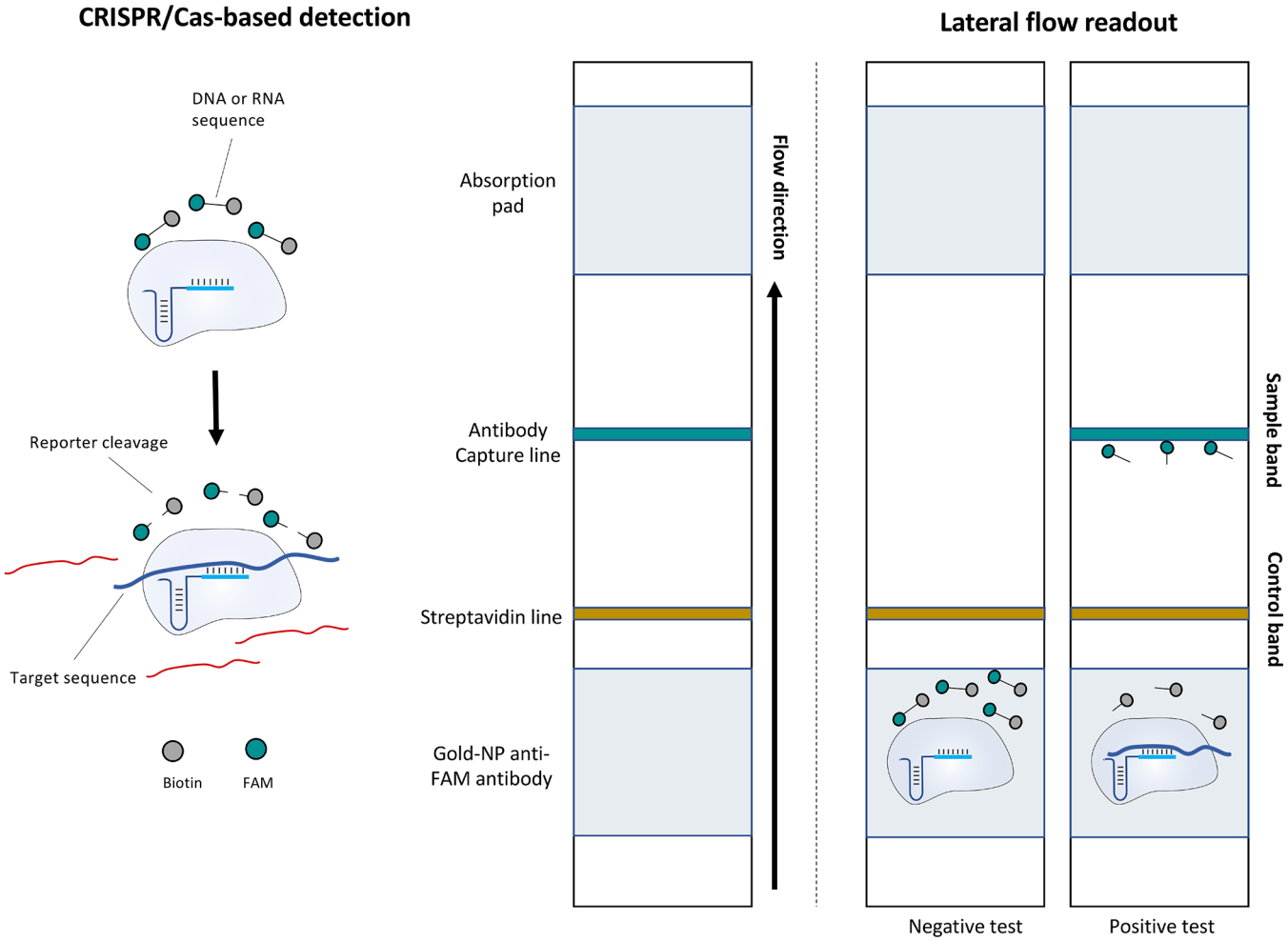

Figure 3. Schematic of lateral flow detection of CRISPR-based biosensing. The CRISPR-based lateral flow detection relies on the cleavage of a FAM-biotin reporter by the collateral activity of the Cas enzymes upon target recognition, allowing for detection on commercial lateral flow strips. The reporter accumulates anti-FAM antibody-gold nanoparticle conjugates at the first line on the strip (brown), preventing binding of the antibody-gold conjugates to protein A on the second line (green); cleavage of reporter would reduce accumulation at the first line and result in signal on the second line.

transcripts. By using reporter RNAs that release a fluorescent signal upon cleavage, the binding of active Cas13 to the targeted RNA will activate the collateral activity of Cas13, leading to the cleavage of reporter RNAs and release of the signal, indicating the presence of the target RNA (Figure 2D).

This collateral activity was the basis for the development of SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) method.[41,42] SHERLOCK harnesses the nonspecific activity of Cas13 to cleave a quenched fluorescent reporter RNA molecule upon target recognition.[42] The SHERLOCK system has been shown to be able to detect specific strains of Zika and Dengue viruses, distinguish between different pathogenic bacteria, to be sensitive to genotype human DNA, and to identify mutations in tumor DNA as well. The power of SHERLOCK was further improved by developing HUDSON (heating unextracted diagnostic samples to obliterate nucleases) that allows SHERLOCK to detect pathogenic nucleic acid directly from biological fluids at very low concentrations (as low as 1 copy/microliter).[43]

Further efforts have continued to improve SHERLOCK for better diagnostics, which resulted in the development of SHERLOCKv2.[25] By utilizing the different specificities of different Cas proteins, including Cas12 and Cas13, SHERLOCKv2 enabled multiplexed nucleic acid detection of at least four distinct targets in a single reaction. In addition, the sensitivity of the system was further increased by harnessing an auxiliary CRISPR-associated enzyme Csm6 that amplifies the signal upon Cas13 collateral activity. To make the system portable and user-friendly, an equipment-free lateral flow system was designed using FAM-biotin reporters.[25]

In the lateral flow system, an RNA or DNA (depending on the Cas effector) reporter containing terminal fluorescein and biotin moieties is used. At one end, the streptavidin binds to biotin and at the other end, the antifluorescein antibodies labeled with gold nanoparticles bind to the fluorescein end of the reporter. If collateral Cas activity is induced, the RNA reporter will be cleaved and gold nanoparticle-labeled antibody will flow to a test line in the lateral flow device, which has an antispecies secondary antibody that will form a colored product at the second line, indicating the presence of the target (Figure 3). This system was successfully used for the detection of Zika and Dengue ssRNA viruses and mutations in patient liquid biopsy samples.[25] In addition, SHERLOCKv2 was harnessed for different agriculture applications, including genotyping and trait quantification.[44]

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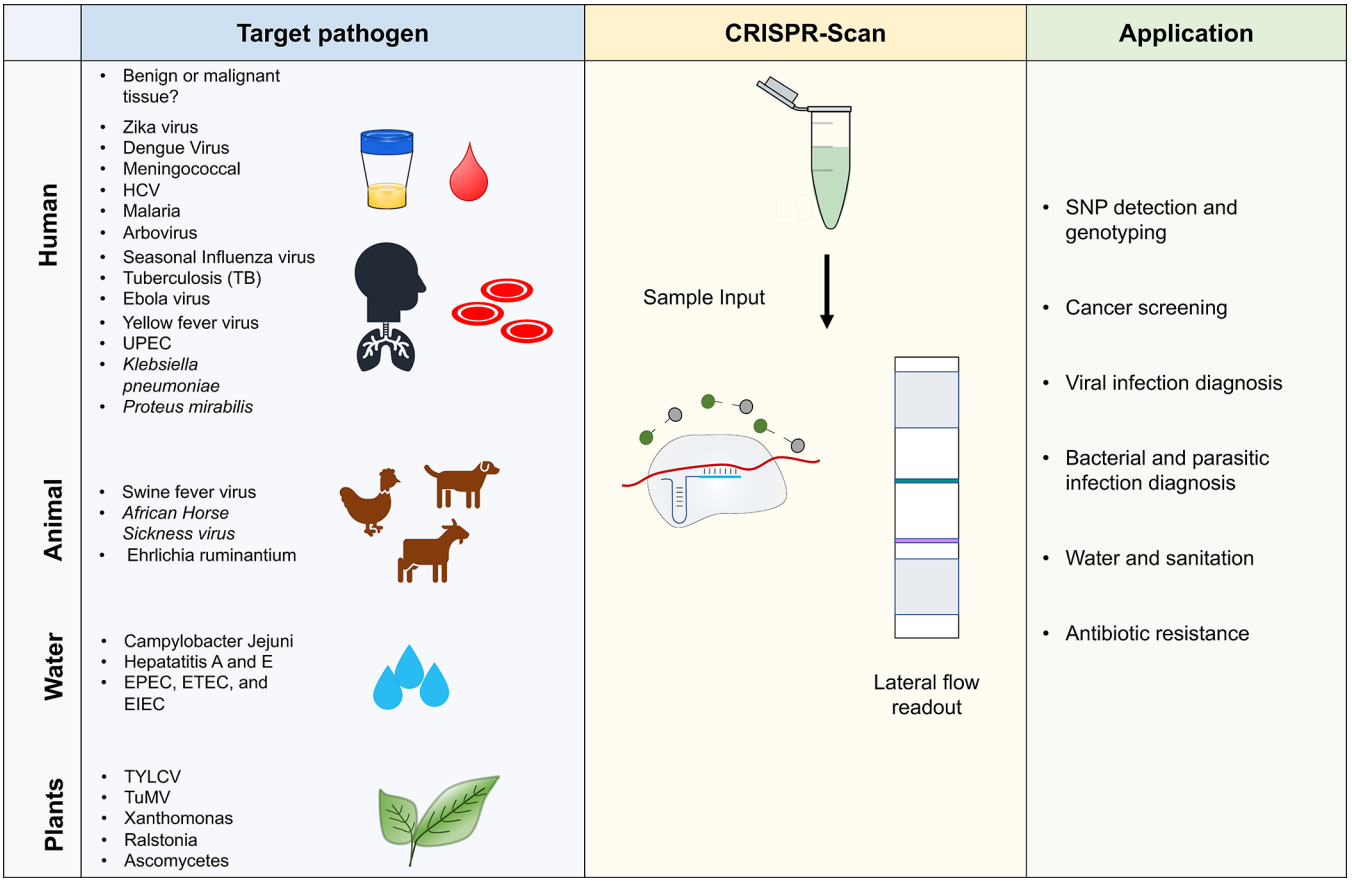

Figure 4. Potential applications of CRISPR-based biosensing platforms. CRISPR-based biosensing platforms can be used for a diverse diagnostic purpose, such as sensing and genotyping pathogenic nucleic acids from different samples including human, animal, water, plant, and other environmental sources. The lateral flow CRISPR-based biosensing allows rapid and sensitive diagnosis of nucleic acids extracted from these various samples. Such a platform enables diverse diagnostic applications in medicine, agriculture, and other research areas.

RESHAPING THE FUTURE OF DIAGNOSTICS VIA CRISPR-BASED TECHNOLOGIES

Timely detection and assessment of pathogen infection is key for effective implementation of disease control and eradication strategies. Therefore, the generation of effective in-field deployable diagnostics will improve the detection, monitoring, and control of infectious diseases. Current in-field diagnostics suffer from major drawbacks including sensitivity, cost, and the need for equipment and skilled technical staff. Newly emerging CRISPR-based diagnostics have the potential to overcome these drawbacks. CRISPR-based biosensing systems could provide a rapid, in-field, sensitive, specific quantitative assay capable of detecting specific sequences, including differentiating single nucleotide polymorphisms (SNPs), and are amenable to multiplexing. Interestingly, different CRISPR/Cas-based diagnostic systems have shown different capabilities of nucleic acid detection at a single-base resolution. For example, a comparison between Cas12a and Cas14 has shown that Cas14 was capable of discriminating a single SNP responsible for the eye color, while Cas12a failed to do so.[20] However, a recently developed Cas12b-based detection method named Cas12b-mediated DNA detection (CDetection) has been shown to distinguish differences at a single-base level, providing an efficient platform for DNA detection at a single base resolution.[29] In addition, the single nucleotide specificity of SHERLOCK has been shown to distinguish different SNPs from different ZIKV pandemics, indicating the high specificity of SHERLOCK to sense and genotype single-nucleotide mutation.[43]

CRISPR-based diagnostics can detect attomolar quantities of the virus nucleic acids, indicating that they are highly sensitive compared to the femtomolar detection range of PCR-based methods.[41,45]

Improvements in diagnostics are decreasing the cost and producing field-ready assays and kits. For example, paper-based diagnostics can cost as little as $0.20 per sensor.[46] Paper-based methods enable easier mass production of the biosensors, which can be stored for over a year at room temperature and can be applied by nontrained staff in locations where laboratory infrastructure is lacking.[47] Moreover, lateral flow systems, which detect antigens using capillary flow of reagents across a cellulose substrate (the most well-known example being the common pregnancy test) can provide sensitive, low-cost detection of the products of CRISPR assays.[48] Because this diagnostic system does not require special instruments or expensive reagents, it represents an efficient, low cost, simple, and easily accessible platform for pathogen nucleic acid detection and other genotyping applications.[49] Such systems are excellent for low cost point-of-care diagnostic purposes. Point-of-care diagnostics must include several features: (1) Allow patients or samples (such as plant tissues or water) to be tested and receive the results rapidly (i.e., during the same visit, or within minutes). (2) The test is conducted near where the patient is receiving care or the sample was collected. (3) The test

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can be conducted and interpreted by nonspecialists. (4) The diagnostic can be delivered in a variety of settings including clinics, fieldwork sites, etc. These tests have several advantages for medical applications; for example, the point-of-care test will simplify the testing process and may provide less opportunity for people to be lost for follow up. Point-of-care tests will also increase the number of people tested, since tests can be administered and interpreted outside of medical facilities and in remote settings. This is especially important for populations who do not have access to health care and for responses to emerging disease outbreaks.[2,50]

CRISPR-based-diagnostics have multiple applications in medicine and beyond. For example, they could be used to determine pathogen genotypes, thereby enabling tailored treatments.[51] They can also be used to detect human, animal, plant, or environmental pathogens. For example, determining the identity and genotype of a plant pathogen could inform the amount and type of pesticide application. Detection of key pathogens could allow rapid evaluation of water quality or assess bioterror threats. They can be used to detect pathogens in food to prevent food-borne illness,[52] and be used for plant seed inspections, testing for genetic modifications, verification of the identity of a food, and myriad other applications (Figure 4).

CRISPR-based diagnostic devices can be easily modified to (in principle) detect any target nucleic acid sequence by changing the gRNA sequence, thereby unlocking the potential to detect pathogens in human, animal, food, and environmental samples. As such these CRISPR/Cas-based diagnostic devices have a great potential to improve human, environment, and plant health with inexpensive, rapid, specific, in-field deployable detection assays.

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Notes

The authors declare no competing financial interest.

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