CRISPR-based nucleic acid diagnostics for pathogens

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Abstract

Pathogenic infections remain the primary threat for human health, especially the global COVID-19 pandemic. It is important to develop rapid, sensitive and multiplexed tools for detecting pathogens and their mutations, particularly the tailor-made strategies for point-of-care diagnosis allowing for use in resource-constrained settings. The rapidly evolving CRISPR/Cas systems have provided a powerful toolbox for pathogenic diagnostics via nucleic acid tests. In this review, we first describe the resultant promising class 2 (single, multidomain effector) and recently explored class 1 (multisubunit effector complexes) CRISPR tools. We present the diverse engineering nucleic acid diagnostics based on CRISPR/Cas systems for pathogenic viruses, bacteria and fungi, and highlight the application for detecting viral variants and drug-resistant bacteria enabled by CRISPR-based mutation profiling. Finally, we discuss the challenges such as the development of preamplification-free diagnostic assays and present the emerging CRISPR systems and CRISPR cascade that potentially enable multiplexed and preamplification-free pathogenic diagnostics.

Keywords: CRISPR/Cas; Craspase; pathogen; SARS-CoV-2 variants; drug resistance

1. Introduction

Pandemic outbreaks, particularly coronavirus disease 2019 (COVID-19) epidemic, highlight the demand to enhance surveillance systems and diagnostic capacities for pathogenic biosafety [1]. The global COVID-19 pandemic has infected 627 million cumulative cases and caused 6.56 million cumulative deaths, and it still results in nearly 80 thousand new cases per day, as of November 1, 2022 (WHO Coronavirus Dashboard, https://covid19.who.int/). Particularly, mutations in viruses spontaneously occur during their replication, and some of them generally account for the variants of concern (VOCs) that could increase the infection severity and render vaccines ineffective [2]. Besides, mutations in bacteria are often the cause of antimicrobial resistance, and horizontal transfer of resistant gene among bacteria is taken as another important way to confer resistance [3]. Development of the diagnostic tools, especially point-of-care testing (POCT), capable to infer pathogens and their mutations is highly important for pandemic control.

Current diagnostic technologies for pathogens mainly dependent on culturing and colony counting, antibody-based assay (enzyme-linked immunosorbent assay, ELISA) and nucleic acid tests (such as quantitative polymerase chain reaction, qPCR) [4,5]. Application limits of these diagnostics lie in high-cost, long turn-around time and the dependent on the sophisticated equipments, hindering their use in resource-constrained settings where generally lack diagnostic reagents and testing infrastructure. Nucleic acid-based technologies, that require only knowledge of the pathogen genome sequence enable accurate and early diagnosis. Combining with nucleic acid preamplification

endows high-sensitivity, moreover, the improvement of assaying specificity, especially sing-mutation resolution, is equally essential for pathogen diagnosis. The clustered regularly interspaced short palindromic repeat (CRISPR)-associated (CRISPR/Cas) system is a promising tool for nucleic acid tests. The evolutionary arms race has led to numerous CRISPR systems with different variations in mechanisms and functionalities. Contributed to the high target sequence specificity and programmability, CRISPR/Cas-based nucleic acid diagnostics have been widely developed for pathogens, and some of which are suitable for on-site detection via integrating with lateral flow paper and mobile phones [6-8].

In this review, we introduced the evolution of the class 1 and class 2 CRISPR/Cas systems as well as the major advances of their applications in nucleic acid diagnostics (Fig. 1). We then presented the applications of CRISPR/Cas-based nucleic acid techniques in pathogenic diagnostics, mainly including viruses, bacteria and fungi. Particularly, we highlighted the application for detecting viral variants and drug-resistant bacteria enabled by CRISPR-based mutation profiling. The challenges such as the development of preamplification-free assay and the potential of CRISPR cascade and new emerging CRISPR systems have been discussed.

2. CRISPR toolboxes

CRISPR/Cas systems pose important roles in the adaptive immune system, and are widely discovered in about 87% archaea and 47% bacteria [9]. CRISPR/Cas systems function as the highly specific molecular scissors to shear invading nucleic acids. A surveillance CRISPR/Cas scissor primarily depend on a ribonucleoprotein

assembled from a Cas effector complex and a single-guide RNA (sgRNA) composed of a non-coding CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA) [10]. According to the difference of Cas genes and their proteins, CRISPR/Cas systems were divided into 2 distinct classes, 6 types [11]. Class 1 systems have multisubunit effector complexes composed of multiple Cas effectors, some of which are responsible for pre-crRNA processing, some for crRNA binding and some for nuclease cleaving, whereas all functions in class 2 systems are executed by only a single Cas effector (Fig. 2). Class 1 systems contain type I, III and IV, while in class 2 systems there also have three subtypes: type II, V and VI. Several promising CRISPR/Cas variants mainly in class 1 systems have been engineered, including a series of dead Cas effector and other mutants represented by Cas9 (H840A) and Cas9 (D10A) [12].

2.1. Class 1 CRISPR toolbox

A typical feature in class 1 system that distinguishes from class 2 is the assembly of multisubunit effector complexes (the CRISPR-associated complex for antiviral defence). The mutisubunit effector module consists of Cas3, Cas5-Cas7, Cas8, Cas10 and Cas11, in different class 1 combinations, the effector module contains different Cas proteins relying on the types and subtypes (Fig. 2). Additionally, the upstream pre-crRNA processing in class 1 system is directly catalyzed by Cas6, whereas in subtype I-B/C systems, Cas5 seems to functionally replace Cas6 [13]. All the class 1 subtypes by far have been employed in developing nucleic acid diagnostic technologies except for type IV, therefore, we next focus on the type I and III.

Class 1 type I system is further divided into 7 subtypes (I-A to I-G) [11]. The

signature *Cas3* gene (or variant *Cas3'*) existed in the whole of type I loci. *Cas3* gene encodes a helicase-nuclease fusion enzyme: the helicase domain with a demonstrated capacity to dissociate double-stranded (ds) nucleic acids (dsDNA or RNA-DNA duplexes) could be stimulated by a single-stranded DNA (ssDNA) [14,15]; another fused N-terminal HD nuclease domain is involved in the nick of the non-target strand (NTS) DNA and the processive degradation of the dsDNA near the PAM, and the further robust collateral cleavage upon ssDNA [16]. Type 1 system typically functions to degrade DNA in a two-step process: (i) Cascade-mediated identification of the DNA target complementary to crRNA; (ii) target binding-triggered DNA degradation in *trans* caused by the recruited Cas3 [17]. Therefore, the signal reporting of nucleic acid diagnostics using type I systems generally relies on the cleavage activity of Cas3 effector.

Two recent works have developed nucleic acid diagnostics using type I system. The well-defined subtype I-E system was used to develop a cost-efficient and equipment-free nucleic acid sensing assay, named Cas3-Operated Nucleic Acid detection (CONAN) [18]. Coupling LAMP preamplification with multiple turnover collateral cleavage of ssDNA signal probes by activated Cas3 endows the CONAN assay with a high-sensitivity. It is recently proved that Cas3 and subtype I-A Cascade assemble into an integral effector complex, distinct from the *trans*-recruitment of Cas3 in other type I systems [17]. And preamplification-free nucleic acid detection can be achieved using this subtype I-A system, and it enabled a pM-sensitivity for dsDNA or ssDNA target within 15 min, while targeting ssRNA obtained a 100-fold lower

sensitivity. By contrast, RNA/DNA heteroduplex target requiring at micromolar range could be detected. Moreover, the use of the lateral strip assay in both work has no trade-off in sensitivity.

Class 1 type III system targets RNA and consists of subtypes A to E [19,20]. Subtype III-A through III-D systems encompass the signature gene *Cas10* — also known as Csm1 in subtype III-A/D and Cmr2 in subtype III-B/C systems [21,22]. Cas10 protein, the largest subunit of type III effectors, harbors multiple domains (a HD domain responsible for collateral DNase, two Palm domains for nucleic acid cyclases, and two small α-helical domains) [23]. The multisubunit effector complex of the type III system comprises Cas10 and additional Cas proteins (Csm2-5 in subtype III-A/D or Cmr1 and Cmr3-6 in subtype III-B/C) [24]. Corresponding to Cas3 protein of type I system, Csm6, an auxiliary CRISPR-associated ribonucleases, is responsible for the signal reporting in type III system. Different from the physical association Cas3 with Cascade, the linking of Cas10-containing effector for invader sensing to Csm6 ribonucleases depends on the Cas10-catalysed cyclic riboadenosines which can act as the activator for Csm6 [25]. Target RNA binding subtype III-A system shows 4 enzymatic activities: (i) specific *cis*-cleavage upon target RNA by Csm3; (ii) collateral DNA degradation by Cas10; (iii) synthesis of cyclic riboadenosines (cOA) by Cas10; (iv) collateral RNA cutting in trans by the cOA-activated Csm6. In subtype III-B system Cas10 (Cmr2) subunit lacks the HD nuclease domain, and hence lacks the DNase activity [26]. The recently identified subtype III-E is an atypical type III system. Different from other type III system, subtype III-E encodes a single protein (knew as

gRAMP and Cas7-11) with several domains including one Cas11-like domain, one big insertion domain and four Cas7-like domains (Cas7.1-Cas7.4) [27,28]. Cas7-11 effector cleaves the target RNA complementary to the crRNA at two defined positions. And similar to Cas12 and Cas13, Cas7-11 possesses pre-crRNA processing activity without the need for additional enzymes. Notably, it is identified that Cas7-11 can associate with TPR-CHAT protease Csx29 (a caspase-like protein) to assemble into Craspase (CRISPR-guided caspase) that capable of RNA-activated cleavage of Csx30 [29-31]. The RNA-activated protein cleavage using Craspase can be explored to sense RNAs either in vitro or inside cells[32].

Several nucleic acid sensing strategies were reported by utilizing different auxiliary CRISPR-associated enzymes, all of which can be activated by cOA with ring sizes ranging from cA_3 to cA_6, such as Csm6, Can1, Can2 and NucC [25,33-37]. A particular type III-A system present in *Lactobacillus delbrueckii* subsp. *bulgaricusthe*, termed LdCsm, is capable of ssDNA cleavage while lacking the cOA signaling pathway [37]. LdCsm enabled sensitive RNA detection at the concentration of 10 nM target and a single-nucleotide resolution based on the only ssDNase. Sridhara et al. developed a MORIARTY assay (Multi-pronged, One-pot, target RNA-Induced, Augmentable, Rapid, Test sYstem) utilizing both the target-triggered ssDNase and cOA signaling pathways [36]. Multiplex signaling conferred the MORIARTY assay with a higher sensitivity than that using single signaling pathway. In addition, three detectable Cas10-mediated polymerase products enabled to turn out multiple readouts [35], in which, cOA signaling, pH change during cyclic nucleotide polymerization and pyrophosphate

sequestrating the metallic ions allowed for fluorometric and colorimetric detection, respectively. Nucleic acid diagnostics using subtype III-B systems were alike to subtype III-A, mainly performing the cOA signaling that generally using TTHB144 [38] and NucC [34]. Furthermore, subtype III-A system from *Thermus thermophilus* (TtCsm) can simultaneously generate cA_3 and cA_4 that can be respectively recognized by NucC and Can2, thus combining with two ancillary nucleases could improve sensitivity of the nucleic acid diagnostic [33].

2.2. Class 2 CRISPR toolbox

Class 2 systems comprise a single large Cas effector protein — Cas9 (subtype II), Cas12 (subtype V) and Cas13 (subtype VI) — with multidomain that responsible for pre-crRNA processing, crRNA binding and nucleic acid cleavage (Fig. 2) [11]. It is the assembly of multiple domains into a single protein that renders them easily usable. Different from Cas6-executing processing in class 1 systems, pre-crRNA processing in type II systems (Cas9) is carried out by the bacterial RNase III — not a Cas protein. Contrastingly in the bulk type V and VI systems, the distinct catalytic domain responsible for processing is naturally fused in the large Cas effector protein (Cas12 and Cas13).

The first use of engineering CRISPR/Cas9 system for specific gene editing in eukaryotic cells opens a brand-new era in biotechnology [39]. In type II effector (Cas9), two endonuclease domains, HNH and RuvC, are responsible for nicking the TS and NTS, respectively, upon target DNA binding the guide RNA (gRNA) [40]. The recognition (REC) lobe of Cas9 protein mediates the recruitment of protein by gRNA,

and the protospacer adjacent motif (PAM)-interacting region recognizes the PAM sequences that is beneficial for the preliminary screening of target [41]. Activated Cas9 nicks target dsDNA precisely at 3-nt upstream (5'-side) of the PAM, generating in a blunt-end break. The highly conservative PAM (*e.g.*, 5'-NGG for *Streptococcus pyogenes* Cas9, SpCas9) offers a further improvement of assay specificity for development of diagnostic tools, however, it greatly limits the target site selection. To overcome this restriction, a SpCas9 variant, named SpGY, with expanded set of NYN PAMs which is a near-PAMless [42], significantly broadening the applications CRISPR/Cas tools in bioanalytical chemistry. Additionally, high demand for diagnostics and gene editing has proliferated a dramatic expansion of class 2 system, including multiple and diverse variants. Commonly used Cas9 variants contain Cas9(H840A) that only nicks NTS, Cas9(D10A) that only nicks TS and dead Cas9 that is nuclease-deficient [12]. Cas9(H840A) or Cas9(D10A) can act as a DNA nickase, allowing for the development of isothermal nucleic acid amplification strategies [43,44].

Type V effector (Cas12, previously known as Cpf1), another DNA targeting system in class 2, possesses a collateral cleavage activity on ssDNA in addition to target dsDNA cleavage in *cis*, while the *cis*-cleavage produces a sticky-end break [45]. Cas12a is the first discovered member in Cas12 family, all enzymes of which share similar structural components. Differently, Cas12a only requires a crRNA for guiding target recognition [46], while more compact Cas12 proteins, such as Cas12b and Cas12f (also known as Cas14a), need both crRNA and tracrRNA. The PAM for Cas12a commonly including *Lachnospiraceae bacterium* Cas12a (LbCas12a) and its *Acidaminococcus*

ortholog (AsCas12a) is 5'-TTTN [46,47], and for *Bacillus hisashii* Cas12b (BhCas12b) is 5'-ATTN [48]. The cleavage of nearby nontarget ssDNA makes Cas12 a promising and powerful signal-amplifying element in nucleic acid diagnostic machinery. In addition, Cas12 can target ssDNA and activate the subsequent collateral cleavage activity with no need for PAM [49,50], further promoting the CRISPR/Cas toolbox for nucleic acid tests.

Cas13 family (type VI effectors) is the only one RNA-targeting system in class 2, functions as a ribonuclease [51]. Until now, 6 subtypes have been identified: Cas13a (formerly C2c2), Cas13b, Cas13c, Cas13d, Cas13X and Cas13Y [11], as well as 5 novel subtypes have been deduced based on metagenomic analysis: Cas13e, Cas13f, Cas13g, Cas13h and Cas13i [52]. Cas13 effector adopts a bilobate structure: the REC lobe acts as the binding site for crRNA, and the NUC lobe composed of two higher eukaryote/prokaryote nucleotide-binding (HEPN) domain plays the RNase activity that catalyzing cleavage of both target ssRNA and bystander nontarget ssRNA. Upon target RNA loading, Cas13 effector undergoes a conformational shift in the NUC lobe to form a stable RNase pocket with the two HEPN domains, which mediates the destruction of invading RNA. RNA-targeting by Cas13 effectors is restricted to the vicinity of a protospacer flanking sequence (PFS).

Nucleic acid diagnostics using the class 2 system have been substantially developed. Cas9 only has the *cis*-cleavage activity that enables no signal amplification, hence generally is coupled with nucleic acid amplification strategies, such as polymerase chain reaction (PCR), rolling circle amplification (RCA) [53], strand

displacement amplification (SDA) [54] and loop-mediated isothermal amplification (LAMP). A Cas9-triggered hairpin probe-mediated biosensing method (termed CHP) for detection of DNA targets was demonstrated utilizing Cas9 as the target recognition element and RCA coupled SDA as the signal amplification element [54]. This CHP assay achieved a aM-sensitivity and a single-nucleotide resolution, the high-sensitivity and specificity conferred the reliable detection of DNA and its mutations without DNA isolation steps in serum samples. Oppositely, the assay using ligation combined with RCA as target recognition and amplification element and Cas9 as the reporting element was also developed, and it achieved a fM-sensitive for microRNA detection [53]. Cas12 and Cas13 effectors both possess multiple turnover *trans*-cleavage activity that can intrinsically realize signal amplification, enabling preamplification-free detection of nucleic acids [55-57]. Targeting multiplex sites can increase activation of Cas12 or Cas13 effectors, benefitting sensitivity improvement [58,59]. Nuclease-dead engineering of Cas effectors coverts them from an RNA-guided nucleases to simple binding proteins. dCas proteins assembled with crRNA provide complementary tools for fluorescence *in situ* hybridization (FISH) using nucleic acid-binding proteins, enriching the toolbox for *in situ* single-cell analysis [60].

2.3. CRISPR Cascade using Class 1 and 2 systems

Attributing to the diverse and abundant CRISPR nucleases, CRISPR cascade strategies were reported in succession [61-65]. Tandem CRISPR nucleases pose the potential for preamplification-free detection with a fM-sensitivity and improvement of the assaying specificity by multiple recognition. The first CRISPR cascade strategy

using Cas12a/Cas12f and Cas13a was constructed for nucleic acid diagnostic [61]. The Cas12f-Cas13a cascade assay showed a 16-fold higher sensitivity (~6.25 fM) than Cas12a-Cas13a cascade one for microRNA. Another high-profile CRISPR cascade strategy for preamplification-free detection of RNA utilized two unrelated CRISPR nucleases, Cas13a and Csm6 [62,63]. The ribooligonucleotide A_4-U_6 could be specifically cleaved by target-activated Cas13a, generating A_4>p with a 2',3'-cyclic phosphate that capable of activating the following Csm6 reporting. Additionally, with the assisted of multiplex PCR or RPA, simultaneously employing multiple Cas effectors with different signaling pathways, such as DNA-targeting Cas12a and RNA-targeting Cas13a, can establish CRISPR cascade capable of multiplex nucleic acid diagnostics. Two CRISPR cascade platforms have been reported to carry out authentication of genetically modified (GM) crops via simultaneous detection of characteristic genes — CaMV35S and T-nos [65], and dual-gene detection of viruses [64].

3. Pathogenic diagnostics

Pathogens, mainly including virus, bacteria and fungi severely threaten human health [66]. Pathogens may have high infectivity and/or toxicity in low doses, For example, the infectious dose of *E. coli* O157:H7 is lower than 100 cells [67]. Notably, genetic mutations in pathogens can potentially lead to an increase of infection risk and severity, and drug-resistant bacteria. Besides, a specific pathogenic variant contains a combination of several mutations, such as the emergent SARS-CoV-2 VOCs —Delta, Omicron [68], and quinolone-resistant *Salmonella* [69]. CRISPR systems have evolved nucleic acid tests related to sensitivity, base-resolution, multiplexing and POCT

feasibility [70]. We will highlight current diagnostic technologies using the CRISPR toolbox for pathogenic viruses, bacteria, fungi, and their variants or mutations, as well as their contributions to pathogenic safety control.

3.1. Viral infection diagnosis

Viruses are small, infectious, non-cellular pathogens composed of DNAs or RNAs that carry genetic information and an encapsulating protein shell, and which can infect human, animals and plants [71]. Nowadays, the outbreak caused by viruses — e.g. Zika virus, african swine fever virus (ASFV), Ebola virus and SARS-CoV-2 — is a persistent threat to public health and global security [72-74]. The mostly recently viral epidemic outbreak is the COVID-19 caused by pathogenic SARS-CoV-2. In addition to person-to-person transmission, SARS-CoV-2 enables person-to-material/material-to-person transmissions [75], highlighting the demand for rapid and accurate diagnostic methods. Therefore, CRISPR/Cas-based nucleic acid diagnostics have frequently reported for SARS-CoV-2, and other reported target viruses include ASFV [59,76], Zika virus [6,73,77], Dengue virus [6], Ebola virus [74] and hepatitis B virus [78].

The integration of CRISRP with nucleic acid preamplification, such as RPA, PCR and LAMP, enabled sensitive nucleic acid diagnostics. Cas9 and its variants incorporated with preamplification strategies allowed the sensitive profiling of viral infection. To achieve low-cost and rapid detection of Zika virus, nucleic acid sequence-based amplification-CRISPR cleavage assay (NASBACC) employed Cas9 to mediate the isothermal NASBA amplification and toehold switch activation that were capable of colorimetric sensing of Zika virus (Fig. 3A) [77]. Additionally, the distinct features

of Cas12 — multiple turnover *trans*-cleavage, capable of targeting both dsDNA and ssDNA and no requirement of tracrRNA — make it emerging as an alternative for Cas9. Doudna group pioneered the development of a Cas12-based rapid and virus-specific diagnostic assay named DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) for human papillomavirus (HPV) diagnosis that yielded an attomolar sensitivity (Fig. 3B) [79]. And DETECTR has been widely applied in other virus diagnosis, including SARS-CoV-2 [80], influenza A and B[81], potexvirus and poteyvirus [82]. Apart from RPA preamplification, assays integrating PCR [83] and LAMP [84,85] into DETECTR were capable of achieving high-sensitivity lower than 5 copies of SARS-CoV-2. Additionally, the viral diagnostic assay replacing Cas12a in DETECTR with RNA-targeting Cas13a, named specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) (Fig. 3C) [6]. SHERLOCK could determine ssRNA of Dengue and Zika virus at femto - and atto-molar sensitivity (~50 fM and ~2 aM), respectively. Compared with amplification strategies for genomic DNA, NASBA, as the unique RNA amplification strategy is more convenient in assisting RNA-targeting Cas13a assay. Wang et al. reported a NASBA-Cas13a assay for label-free detection of SARS-CoV-2 aided by a light-up RNA aptamer, yielding a sensitivity down to 0.216 fM [86].

Moreover, the emerging class 1 CRISPR systems were also reported in nucleic acid assays for pathogenic viruses. The subtype I-E system was utilized to develop a cost-efficient and equipment-free nucleic acid sensing assay (CONAN) for SARS-CoV-2 diagnosis [18]. The optimal type of PAM, LAMP preamplification and multiple

turnover collateral cleavage of ssDNA signal probes by activated Cas3 endowed the CONAN assay with a high-sensitivity as low as 10^2 copies. The foregoing mentioned MORIARTY assay using class 1 type III CRISPR system allowed to detect 2,000 copies/μL SARS-CoV-2 in amplification-free settings, attributing to the simultaneous use of *cis*-ssDNase and cOA signaling pathways [36]. And coupling with RT-RPA preamplification, MORIARTY assay significantly improved the sensitivity to achieve a LOD (limit of detection) of 60 copies/μL. Besides, harnessing the changes in pH and pyrophosphate concentration that occur during cyclic nucleic polymerization could realize the colorimetric or visible fluorometric detection of SARS-CoV-2 [35]. Inspired by the FIND-IT assay using activator variant for TtCsm6 [62], Wiedenheft group adopted the Can2 nuclease to assist the signaling of type III-A CRISPR capture technique [33]. Use of Can2 lacking the ring nuclease activity can sustain the high level of cA_4 activator, contributing to sensitivity improvement. This assay yielded a 90 fM (5×10^4 copies/μL) detection sensitivity for SARS-CoV-2 RNA from nasopharyngeal swab samples without nucleic acid extraction process.

Separate nucleic acid procedures including preamplification and amplicon detection in viral diagnostics, increase the aerosol contamination risk of amplicon. Uracil-DNA-glycosylase (UDG) can clean deoxyuracil bases existed in amplicons while does not act on natural DNA. In a built UCLD assay (Fig. 4A), UDG clearance occurred before the next round of nucleic acid amplification, which can effectively reduce false-positive diagnosis [87]. Using highly specific primers in the UCLD assay can achieve 100-fold higher sensitivity for SARS-CoV-2diagnostic than traditional

LAMP assay. Besides, one-tube reaction can alleviate the contamination risk. However, DETECTR or SHERLOCK testing in one-pot generally has trade-off in sensitivity, due to the exponential interval with affection by activated Cas12a and the unavailing consumption of Cas12a activity in the early stages of preamplification. Alternatively, to render the nucleic acid amplification and the Cas protein activation compatible, Chen et al. adopted a photoactivated crRNA to sequentially activate RPA preamplification and Cas12a reporting reaction for ASFV diagnosis (Fig. 4B), favoring that the both processes did not interfere with each other [88]. After RPA preamplification, photolysis under UV lamp of 365 nm degraded photocleavable ssDNA complementary to crRNA, activating the Cas12a effector. This photocontrolled Cas12a strategy could detect as low as 2.5 copies ASFV within 40 min. Moreover, physical separation is also effective in alleviation of aerosol contamination. Using a disposable tube-in-tube vessel can completely implement the physical separation — the inner tube with two hydrophobic holes containing RPA reagent and the outer tube containing prestored CRISPR reagents [89](Fig. 4C). This strategy was verified by multiple viruses including DNA virus (ASFV) and RNA virus (SARS-CoV-2), and yielded a LOD of 3 copies/μL.

CRISPR/Cas system with intrinsic signal amplification can serve in preamplification-free nucleic acid detection. Multiplex-crRNA and tandem CRISPR/Cas strategies for diagnosis of Dengue virus, SARS-CoV-2 and Ebola virus have been verified to be able to construct sensitive nucleic acid diagnostic assay. The first work using multiplex-crRNA strategy combined two crRNAs to enhance the sensitivity of Cas13a assay for SARS-CoV-2 sensing, and it achieved a LOD of 200

copies/μL over 30 min measurement (Fig. 5A) [58]. Another work harnessing multiplex-crRNA strategy in Cas12a system enhanced 64-time sensitivity of the Cas12a assay than the conventional single-crRNA system, with a LOD of ~1 pM of ASFV genomic DNA (Fig. 5B) [59]. In addition, CRISPR/Cas-only amplification network (CONAN) for hepatitis B virus DNA detection enabled self/cross catalytic cleavage of nucleic acids, favoring isothermally amplified detection (Fig. 5C) [78]. Target DNA activated Cas12a-crRNA1 complex cleavage the blocking ssDNA in the bubble region, subsequently activating the Cas12a-crRNA2 complex for further positive signal output. The blocking ssDNA partly complementary to crRNA2 was labeled with fluorophore and quencher. The self/cross catalytic amplification empowered an aM-sensitive detection of hepatitis B virus DNA. Given that the product of Cas13a can activate the following Csm6 cleavage, the tandem Cas13a-Csm6 detection was constructed and exhibited higher sensitivity (20 nM of Dengue virus ssRNA) than that using Cas13a alone [63]. Furthermore, inspired by that blocking the degradation of Csm6 activator may sustain the high level of Csm6 cleavage, Doudna group modified a single 2'-fluoro (2'-F) at the cleavage site of activator to realize the blocking effect. The design of using tandem CRISPR nucleases enabled an amplification-free detection of SARS-CoV-2 RNA (Fig. 5D) [62]. The tandem assay created rapid (<20 min) and one-step detection with a sensitivity down to 30 copies/μL of SARS-CoV-2 RNA. A DNA walker principle was built as an alternative strategy to amplify Cas12 cleavage reaction. By combining with a DNA walker machine for amplifying the output signal, a preamplification-free Cas13a assay allowed to detect Ebola RNAs with a LOD of 291 aM (~175 copies

RNA/μL).[74]. Nevertheless, design of efficient DNA walker could complicate the nucleic acid assay.

3.2. Viral variant detection

Viral variants may make them competitively fitter in the living environment, and likely bring great risks to human health, such as VOCs of SARS-CoV-2 and the 2009 pandemic influenza A virus (H1N1) [90-92]. Omicron variants of SARS-CoV-2 owns mutations, K417N, T478K, E484A and N501Y that associated with variation of transmissibility and virulence [91]. The N222 variants of H1N1 frequently occurred in severe and fatal cases [92]. The high specificity of CRISPR-based nucleic acid diagnostics enabled the profiling of viral variants with single-nucleotide resolution.

The presence of base mismatch occurring close to PAM region could dramatically reduce the *cis*-cleavage activity of Cas9. Via sgRNA engineering, Cas9 was used to sense mutations in viral RNAs . For example, a CRISPR-FnCas9-based assay mutated the 2^{nd} and 6^{th} positions adjacent to the PAM on the sgRNA enabled to profile mutations in N501Y variant of SARS-CoV-2 (Fig. 6A) [93]. Patient sample testing — mutant type-infected (n = 22) and wild type-infected (n = 37) — showed a 97% accuracy. Combining with lateral flow strips as readouts can realize visual POC diagnostic, Ali et al. employed mutation-containing dCas9/sgRNA complex as a recognition element that specifically bound FAM-label amplicons (Fig. 6B) [94]. This proposed POC diagnostic allowed sensitive detection of multiple SARS-CoV-2 VOCs — Alpha (deletions of residues 69 and 70), Beta (D215G), Delta (P481R and L452R) and D614G (common to Alpha, Beta, and Delta variants). By incorporating artificial mutations in crRNA, a

highly sensitive Cas12a-based assay has been constructed to identify D614G mutant [95]. Apart from specificity implemented by Cas effectors, ligation reaction can also be used to discriminate SNPs. Target-templated ligation possesses the capacity of single-mutation discrimination. Wang et al. employed the ligation and transcription to produce the D614G-specific DNA templates that capable of triggering the subsequent transcription. The RNA transcripts can activate the multiple turnover cleavage of Cas13a to further amplified the signal. The adoption of broccoli, a light-up RNA aptamer, as the reporter achieved a label-free detection. This assay yielded a high-sensitivity with a LOD of 82 copies of SARS-CoV-2 (Fig. 6C) [96].

Viral variants are commonly caused by multiple mutations, and infections could be caused by multiple viruses. For diagnostic of Omicron with multiple mutations, Liang et al. refined RT-PCR DETECTR to sensitively sense multiple Omicron SNPs via using engineered two crRNAs that separately covered three mutant sites (S371L, S373P, S375F for crRNA-S-37 and Q493R, G496S and Q498R for crRNA-S-49X) (Fig. 6D) [83]. The cover of 3-4 mutations evaded the requirement of utilizing multiple crRNAs. A sensitivity of 2 copies per reaction of the Omicron plasmid DNA has been achieved, and this method specifically differentiated Omicron variants from Alpha, Beta and Delta in a test of 57 positive clinical specimens. The combination of multiple RT-PCR and RT-RPA with CRISPR reporting was demonstrated to be able to realize simultaneous detection of multiple SARS-CoV-2 VOCs (Fig. 6E) [97]. In the assay, the Alpha-and Delta-specific single-nucleotide variants (SNVs) including S982A, D614G and D950N were able to be identified by PAM motif or seed regions of Cas12a system.

Employing a smartphone readout device could detect the SNP-involved SARS-CoV-2 virions with a detection limit of 0.5 copies/mL. Thus, this method is promising for high-throughput screening of SARS-CoV-2 infection both in clinical laboratories and POC test settings. In addition, to address the intolerance of Cas proteins to LAMP temperature (55-65 ℃), a thermostable Cas12b (BrCas12b) from *Brevibacillus sp.*SYP-B805 was selected, thereby readily enabling one-pot BrCas12b-LAMP detection [98]. The one-pot BrCas12b assay showed 96.7% accuracy, 99.4% specificity and 92·8% sensitivity in a test of saliva samples for SARS-CoV-2 VOC discrimination including Alpha, Beta, Gamma, Delta and Omicron. Analogous to BrCas12b, selected *Thermoclostridium caenicola* Cas13a (TccCas13a) remains high thermal stability at LAMP reaction temperature, even possesses robust cleavage activity at a broader temperature (37-70 ℃). This one-pot TccCas13a assay has been demonstrated to detect different SARS-CoV-2 variants [99]. The highest multiplexing assay for pathogenic viruses and their variants was reported by Sabeti group [100]. The assay, termed CARMEN, combined Cas13-based nucleic acid assays with a microwell array which was designed for pairing droplets of amplified samples and sample testing (Fig. 6F), enabling to simultaneously distinguish 169 viruses associated with human health, and multiplex discrimination of dozens of human immunodeficiency virus mutations that are resistant to drug. This assay promises the comprehensive testing of scalable samples, however, not applicable for POCT due to the involvement of the complexing and costly microwell array.

3.3. Bacterial infection diagnosis

Bacteria cause outbreaks of serious infectious diseases. Particularly, the abuse of antibiotics has resulted in increasing prevalence of antimicrobial resistance in both the hospital settings and communities [101].

Combining with preamplification generally confers high-sensitivity to CRISPR/Cas-based diagnostics. A PCR preamplification-based Cas12a assay was reported to detect *Vibrio parahaemolyticus* (*V. parahaemolyticus*) with a LOD of 1.02 $\times 10^2$ copies/μL [102]. Isothermal preamplification can bypass the use of thermal cyclers without compromising the sensitivity. RPA-based Cas12a assay has been used to detect pathogenic bacteria, and enabled sensitivity as low as 101 CFU/mL for *Salmonella* in egg samples [103], 10 copies of plasmid DNA for *S. aureus*, *E. coli*, and *Listeria monocytogenes* (*L. monocytogenes*) [104], 2 copies/reaction of plasmid DNA for *Brucella* spp. in milk[105] and 2.5×10^3 CFU/mL for *Xanthomonas oryzae* pv. *oryzae* in rice [106]. Using gold nanoparticles (AuNPs) as the reporter of CRISPR assays allowed colorimetric visual detection, favoring on-site detection. The Cas12a-AuNPs assay empowered the detection of *Salmonella* with a single-cell sensitivity (Fig. 7A) [107]. In addition, integrating with G-quadruplex deoxyribozyme (DNAzyme) that can catalyse 3,3',5,5'-tetramethylbenzidine sulfate (TMB) to oxTMB could achieve colorimetric bioassays. Yin et al. integrated the G-quadruplex with CRISPR to construct a colorimetric detection, and used a smartphone as the signal readout, contributing to a convenient POCT (Fig. 7B) [108]. This Cas12a-based visual assay can detect *Salmonella* as low as 1 CFU/mL.

Unculturable viable but non-culturable bacteria can escape detection by

conventional culture and colony counting methods. DNA-targeting diagnostics fail to identify whether the bacteria are alive due to that genomic DNA is hard-to-degrade [109]. Oppositely, RNA-targeting diagnostics can accurately indicate live status of bacteria through timely sensing cellular RNA, because of the rapid degradation of RNA in dead cells. NASBA preamplification and Cas13a reporting were incorporated to construct a viable bacterial sensing platform. Dual signal amplification by NASAB and Cas13a reporting endowed a high-sensitivity down to 1 CFU and 1% viable *Salmonella* (Fig. 7C) [110].

To evade carryover contamination that often occurs in nucleic acid amplification-based assays, it is feasible to perform one-pot detection. Through the optimization of reaction conditions such as the concentrations of reagents, a RPA-Cas12a-based one-pot assay without extra sample purification was constructed for detecting *S. aureus* and *E. coli* O157:H7, which yielded a single-cell sensitivity [111]. Alternatively, Cas9 with durable *cis*-cleavage can be applied to degrade undesired amplicons that can lead to cross-contamination. The CUT-LAMP (contamination-free LAMP) assay adopting the above strategy, alleviated carryover contamination. It yielded negligible false-positive results even with contaminants up to 10 pg while traditional LAMP assay had high background in the presence of 1 fg of contaminants (Fig. 7D) [112]. The CUT-LAMP reaction can reliably sense *Salmonella* genomic DNA down to 100 copies, and with no background amplification. Cas12a system for endpoint record of CUT-LAMP products was further tested, it could discriminate the *Salmonella* and *S. aureus* LAMP products. UDG could replace the Cas9 to serve as the cleaner for cross-contaminants. Qian et al.

used the UDG degradation to eliminate the cross-contamination [113]. Cas12a can recognize uracil-rich sequences including PAM sequence of 5'-UUUN, enabling the establishment of a UDG-assisted LAMP-Cas12a assay that allowed the alleviation of cross-contamination. This assay was successfully applied to diagnose the infection of *Candidatus* Liberibacter *asiaticus* associated with citrus huanglongbing[114], and yielded a detection limit less than 10 copies.

Combining with functional nucleic acids could achieve pathogenic diagnostics free of preamplification [115,116]. For example, Deng group reported an assay via CRISPR in tandem with RNA-cleaving DNAzyme, named CRISPRzyme assay (Fig. 7E) [117]. Dual amplification via multiple-turnover cleavage of Cas12a and DNAzyme enables preamplification-free detection. The sequential CRISPRzyme assay yielded a LOD of 62 CFU, 86 CFU and 82 CFU for *V. parahaemolyticus*, *Salmonella typhimurium* (*S. typhimurium*) and *L. monocytogenes*. It was further applied to screening antimicrobial probiotics against fish pathogen — *V. parahaemolyticus*, and found that *Bacillus* and lactic acid bacteria isolated from fermented food could serve as potential antimicrobial probiotics. The reduction of background signal would contribute to the improved sensitivity of CRIPSR-based assay. To obtain an ultralow background, PPCas12a assay designed a pair of DNA reporters labeled with proximal fluorophore and quencher, enabled bacterial detection without preamplification [56]. The proximity of fluorophore to quencher resulted in an ultralow background, favoring that Cas12a directly detect *Salmonella* with a LOD of 619 CFU. The assessment of qPCR indicated that the PPCas12a assay can reliably detect *Salmonella* contamination in complex food

matrices.

3.4. Drug-resistant bacteria detection

Overuse of the antibiotics has led to the widespread prevalence of drug-resistant bacteria. The resultant antimicrobial resistance in bacteria is often attributed from horizontal transfer of resistant gene among bacteria and gene mutations [3]. Methicillin-resistant *S. aureus* (MRSA) is one of the most important horizontally acquired drug-resistant strain [118]. A FISH-like CRISPR assay using dCas9/sgRNA complex as recognition and binding element was reported for visual detection of MRSA [119]. The 10-fold higher output signal of MRSA than that of methicillin-susceptible *S. aureus* (MSSA) suggested that the FISH-like dCas9 assay was capable of accurate identification of bacteria genotypes. To achieve preamplification-free detection Suea-Ngam et al. combined sensitive electrochemical readout with silver metallization to construct a Cas12a-based biosensor to detect the *mecA* gene of MRSA (Fig. 8A) [120], with a quantification limit of 10 fM and a linearity ranging from 10 fM to 0.1 nM. No trade-off in detection performance was observed when detect MRSA in human serum. Drug-resistance induced via genetic mutation is challenging to be detected. Cas12a-signaling amplification refractory mutation system (ARMS)-PCR, called cARMS assay was capable of specific differentiation of SNP-involved *Salmonella* via adopting an allele-specific primer with artificial mismatch (Fig. 8B) [121]. The dual-recognition processes — allele-specific primed polymerization and Cas12a/crRNA hybridization — conferred the single-nucleotide discrimination capacity. And the dual amplification of PCR and Cas12a reporting rendered the cARMS assay a high-sensitivity as low as

~0.5% drug-resistant strain comparable to qPCR. CRISPR/Cas-based diagnostics for identification of SNP-involved bacteria could combine with isothermal amplifications, which can facilitate POCT application.

3.5. Fungal diagnosis

Diseases caused by pathogenic fungi have become a significant issue in both agricultural production and public health security. Increasing nucleic acid diagnostics based on CRISPR/Cas has been developed. The widespread prevalence of wheat scab in wheat-growing period remarkably reduces the wheat yield, and is accompanied by generating varieties of mycotoxins to contaminate food [122]. *Fusarium graminearum* (*F. graminearum*) is the primary causative agent of wheat scab. The integration of Cas12a with PCR enabled the early detection of *F. graminearum* infection (Fig. 9A) [123]. This assay can detect *F. graminearum* with a LOD of 1 fg/μL total DNA that contributed to successful diagnostic of 4-day *F. graminearum* infection. Furthermore, the diagnosis of invading fungal diseases highlighted the need in developing field-deployable POC testing. Assisting with lateral-flow strips to output visual reading, Cas12 integrated RPA assay enabled rapid (<1 h) POC diagnostic of citrus scab with a high-sensitivity down to 1 fg crude DNA [124]. To construct a colorimetric readable assay, Liu et al. developed a PCR-Cas12a-based photothermal assay by employing AuNPs as an carrier for loading horseradish peroxidase (HRP) and ssDNA — they are integrated into the Cas12a reporting system (Fig. 9B) [125]. This assay enabled colorimetric nucleic acid diagnostic of *Alternaria* genes that encoding the citrus brown spot, and capable of identifying targets from citrus, tomato and apple. Nevertheless, the

on-site use of these assays is hindered by the need of nucleic acid extraction which can hardly be proceeded in the field. Wei group reported a rapid nucleic acid extraction process using microneedles, allowing to obtain pathogenic DNAs within l min [126]. The simple sample-pretreatment protocol would facilitate the CRISPR-based nucleic acid tests for in field diagnostics of crop health.

4. Conclusion and outlook

CRISPR-based nucleic acid diagnostics for pathogen infection have been advanced in related to sensitivity, base-resolution, multiplexing and POCT feasibility. However, there are several issues remaining in CRISPR-based tools to be improved. Firstly, a challenge of CRISPR-based nucleic acid diagnostics is the unavoidable use of preamplification for femtomolar targets, particularly in developing POC testing. Preamplification imposes a burden on assaying simplicity, timeframe and aerosol contamination. Strategies using tandem CRISPR nucleases, and CRISPR with other catalytic molecules (such as enzymes and DNAzymes) have been reported to substitute nucleic acid amplification, and are promising to construct preamplification-free assays. Particularly, the recently reported Craspase [29], an RNA-activated CRISPR protease that differs from conventional Cas effectors with nuclease function, has the potential for amplified detection via using protein reporters with high catalytic activities. Second, the improvement of assaying specificity capable of single-nucleotide discrimination is critical for diagnosis of pathogenic variants and mutations. Innovation of the crRNA design, such as chemical modification and mutational introduction would be useful to improve the specificity. Additionally, an engineered split Cas12a has the potential for

nucleic acid diagnostics [127]. The split Cas12a could be fused with two DNA handles by covalent linkage at two ends respectively [128], and the assembly of split Cas12a depends on the specific binding between the two handles and the target sequence. This target-specific assembly of Cas12a may contribute to the improvement of assaying specificity. Third, the multiplexing assay is demanded for confidently profiling pathogenic infections via screening possible pathogens and their variants. To this end, efforts to implement multiplexing Cas effectors and cooperate with microfluidic devices may be considered. Based on the found of Craspase, different reporting approaches — nucleic acid reporter and protein reporter — could be integrated. We can further excavate the tools in the class 1 CRISPR system, which may be explored in multiplex nucleic acid diagnostics [129]. Finally, the difficulty in transport and storage of bioreagents such as Cas enzymes and crRNAs makes the tests not feasible for POCT applications. Reagent storage strategies including lyophilization, nanocarriers and hydrogel are promising for bioreagent preservation. Particularly, the emerging so-called "self-contained" microfluidic devices, which can pre-store all necessary reagents in premeasured quantities, have the potential for constructing mix-and-read and stabilized assays that suitable for POCT [130]. Enrichment and evolution of CRISPR toolbox will facilitate nucleic acid diagnostics and pathogenic biosafety control.

Declaration of competing interest

The authors declare no conflict of interest.

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FIGURE CAPTIONS

Fig. 1. Overview of CRISPR toolboxes and their applications in nucleic acid diagnostics for pathogens. Class 1 and class 2 CRISPR/Cas system, and engineered CRISPR — Craspase and split Cas effector are designed for detecting viruses, viral variants, bacteria, fungi and mutated drug-resistant pathogens.

Fig. 2. Classification of CRISPR/Cas system. Class 1 (multisubunit effector complex) contains type I, III and IV; class 2 (single, multidomain effector) contains type II, V, and VI.

Fig. 3. Preamplification-based nucleic acid diagnostics for viruses. (A) Cas9-based NASBACC assay for Dengue and Zika virus detection [77]. Employing Cas9 to mediate the isothermal NASBA amplification and toehold switch activation allows the visual detection of Dengue and Zika virus. (B) Schematic of DETECTR strategy using Cas12 [79]. HPV viral DNA is amplified by RPA, followed by Cas12a-mediated reporting. (C) Cas13-based SHERLOCK assay for detecting Dengue and Zika virus [6]. Target DNA or RNA is amplified by RPA or RT-RPA, then the RPA amplicons yield RNA transcripts via *in vitro* transcription, which can activate Cas13a trans-cleavage activity for fluorescent reporting.

Fig. 4. Contamination-free nucleic acid diagnostics based on preamplification for viruses. (A) Schematic of UCLD-platform. UDG can clean deoxyuracil bases existed in amplicons while does not act on natural DNA, favoring CRISPR-LAMP platform for contamination-free and amplified detection [87]. (B) Schematic of photocontrollable RPA-Cas12a assay [88]. After enough RPA preamplification,

photolysis under UV lamp of 365 nm degrades photocleavable ssDNA complementary to crRNA, activating the Cas12a effector. (C) Schematic of one-pot Cas13a assay using a disposable tube-in-tube vessel to separate RPA preamplification and Cas13a reporting [89].

Fig. 5. Preamplification-free nucleic acid diagnostics for viruses. (A) Schematic of multiplex-crRNA strategy using Cas13a fluorescent assay for SARS-CoV-2 detection [58]. (B) Schematic of multiplex-crRNA strategy using Cas13a fluorescent assay for ASFV detection [59]. (C) Schematic of CRISPR/Cas-only amplification network (CONAN) for amplified detection of hepatitis B virus [78]. Target DNA activated Cas12a-crRNA1 complex cleavage the blocking ssDNA at the bubble region, subsequently activating the Cas12a-crRNA2 complex for further positive signal output. (D) Schematic of CRISPR tandem assay using Cas13a and Csm6 [62]. Activated Cas13a can cleavage A_4-U_6 to produce A_4>p that activate the subsequent Csm6 cleavage, achieving cascade signal amplification.

Fig. 6. CRISPR/Cas-based nucleic acid diagnostics for virus variants. (A) Schematic of CRISPR-FnCas9-based assay [93]. Artificial mutating the 2^{nd} and 6^{th} positions adjacent to the PAM on the sgRNA to achieve the single-nucleotide specificity for N501Y variant of SARS-CoV-2. (B) Schematic of dCas9-based lateral flow assay [94]. Using dCas9 as a recognition element that specifically bound FAM-label amplicons and lateral flow strips as readouts, and combining with variant-specific sgRNAs for detecting multiple SARS-CoV-2 VOCs. (C)

Schematic of the ligation-triggered Cas13a assay for D614G discrimination. Target-triggered ligation can generate a transcription template with a sequence that the corresponding RNA transcript is capable of activating the subsequent Cas13a reporting. [96]. (D) Schematic of a refined RT-PCR DETECTR for multiply sensing Omicron SNPs [83]. A crRNA contains multiple specific mutant sites, enabling simultaneous detection of multiple mutations. (E) Schematic of simultaneous detection of multiple SARS-CoV-2 VOCs [97]. This assay combines multiple RT-PCR and RT-RPA with CRISPR reporting. (F) Schematic of combinatorial arrayed reactions for multiplexed evaluation of nucleic acids (CARMEN) assay for scalable and massively multiplexed detection of pathogenic viruses [100].

Fig. 7. CRISPR/Cas-based nucleic acid diagnostics for bacteria. (A) Schematic of the PCR-Cas12a-AuNPs assay empowers the detection of *Salmonella* with single-cell sensitivity [107]. Using gold nanoparticles (AuNPs) as the reporter of CRISPR assays allows colorimetric visual detection. (B) Schematic of RPA-Cas12a and G-quadruplex-based colorimetric detection of *Salmonella* [108]. G-quadruplex serves as the reporter of Cas12a system, which can produce a turn-off colorimetric signal. And use of a smartphone as the signal readout can achieve a convenient POCT application. (C) Schematic of the NASBA-based Cas13a assay for viable bacteria sensing [110]. NASBA preamplification produces abundant RNA transcripts that can activate the following Cas13a reporting. RNA sensing can indicate the living status of bacteria. (D) Schematic of a CUT-LAMP for detecting

Salmonella assay [112]. Using Cas9 as the scissor for cleaving undesired amplicons to eliminate cross-contamination. (E) Schematic of the Cas12a-DNAzyme cascade assay for rapid detection of *V. parahaemolyticus* colonized in fish (*perca fluviatilis*) [117]. Target-activated Cas12a can degrade the enzyme strand of DNAzyme, suppressing the DNAzyme reporting.

Fig. 8. CRISPR/Cas-based nucleic acid diagnostics for drug-resistant bacteria. (A) Schematic of electrochemical Cas12a biosensor for detection of *mecA* gene of MRSA via silver metallization [120]. (B) Schematic of the cARMS assay for detection of SNP-involved drug-resistant *Salmonella* [121]. PCR primer with specific artificial mutations enables single-nucleotide resolution, and the Cas12a reporting can enhance both the specificity and signal output.

Fig. 9. CRISPR/Cas-based nucleic acid diagnostics for fungi. (A) Cas12a-based PCR assay for the diagnostic of *Fusarium graminearum* infection of wheat [123]. Target DNA was amplified by PCR, followed by Cas12a reporting. (B) Schematic of a PCR-Cas12a-based photothermal assay for colorimetric detection of *Alternaria* genes that encoding the citrus brown spot from citrus [125]. Coupling magnetic separation with the adoption of HRP-loaded AuNPs as the Cas12a reporter can achieve a visual detection.

Fig. 1.

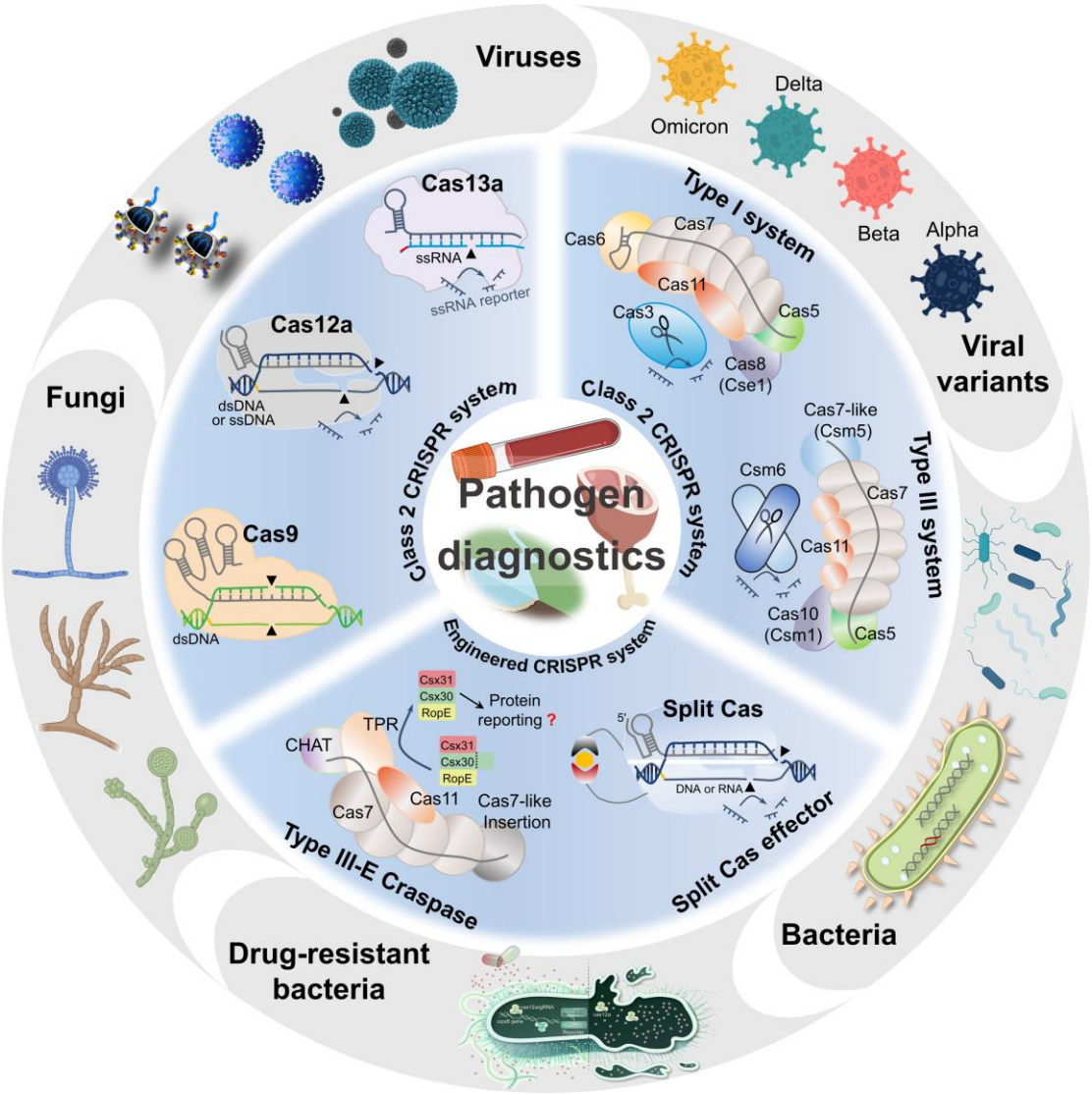

Viruses
Omicron
Delta
Beta
Alpha
Viral variants
Fungi
Bacteria
Drug-resistant bacteria
Cas13a
ssRNA
ssRNA reporter
Cas12a
dsDNA or ssDNA
Cas9
dsDNA
Type I system
Cas6
Cas7
Cas11
Cas3
Cas5
Cas8 (Cse1)
Type III system
Cas7-like (Csm5)
Csm6
Cas7
Cas11
Cas10 (Csm1)
Cas5
Class 2 CRISPR system
Class 2 CRISPR system
Pathogen diagnostics
Engineered CRISPR system
Csx31
Csx30
RopE
Protein reporting ?
TPR
CHAT
Cas7
Cas11
Cas7-like Insertion
Type III-E Craspase
Split Cas
5'
DNA or RNA
Split Cas effector

Fig. 2

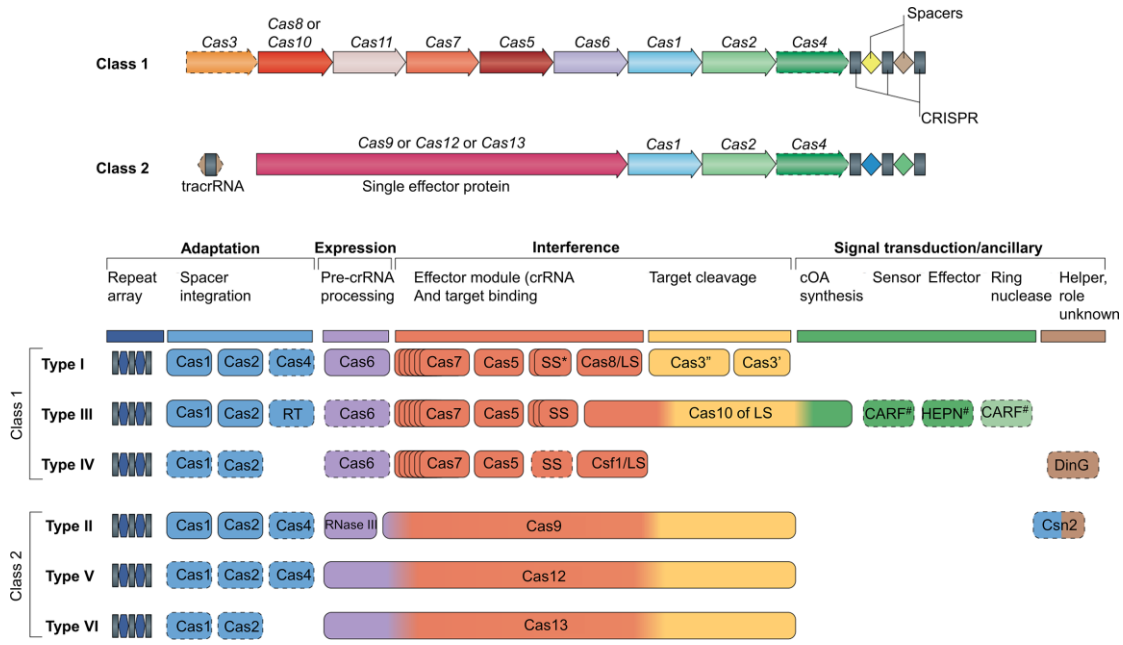

Class 1
Cas3
Cas8 or Cas10
Cas11
Cas7
Cas5
Cas6
Cas1
Cas2
Cas4
Spacers
CRISPR
Class 2
tracrRNA
Cas9 or Cas12 or Cas13
Single effector protein
Adaptation
Expression
Interference
Signal transduction/ancillary
Repeat array
Spacer integration
Pre-crRNA processing
Effector module (crRNA And target binding
Target cleavage
cOA synthesis
Sensor
Effector
Ring nuclease
Helper, role unknown
Type I
Type III
Type IV
Type II
Type V
Type VI
Cas1
Cas2
Cas4
RT
Cas6
Cas7
Cas5
SS*
SS
Cas8/LS
Csf1/LS
Cas3"
Cas3'
Cas10 of LS
CARF#
HEPN#
DinG
RNase III
Cas9
Csn2
Cas12
Cas13

Fig. 3

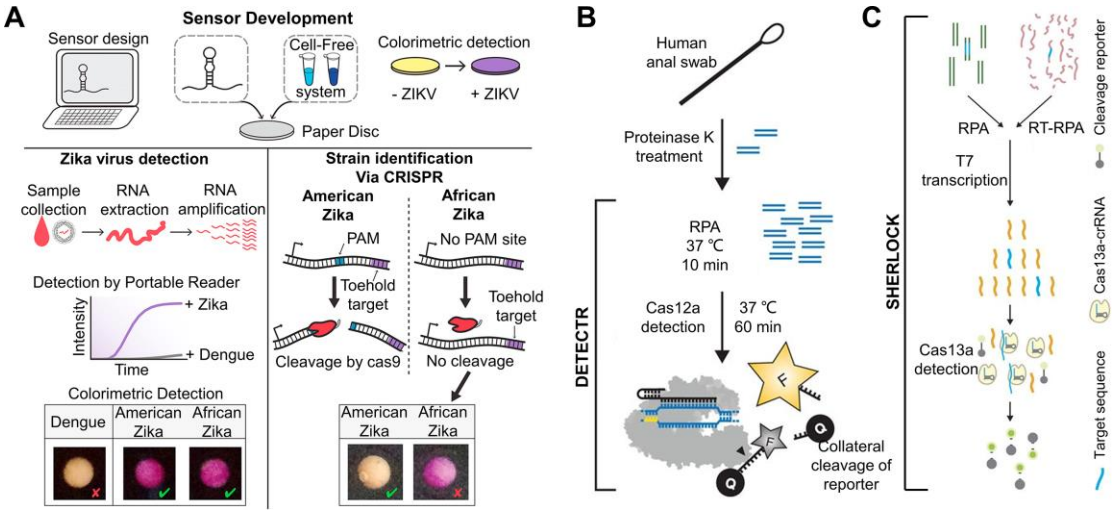

A
Sensor Development
Sensor design
Cell-Free system
Colorimetric detection
- ZIKV
+ ZIKV
Paper Disc
Zika virus detection
Sample collection
RNA extraction
RNA amplification
Detection by Portable Reader
Intensity
+ Zika
+ Dengue
Time
Colorimetric Detection
Dengue
American Zika
African Zika
Strain identification Via CRISPR
American Zika
African Zika
PAM
No PAM site
Toehold target
Toehold target
Cleavage by cas9
No cleavage
American Zika
African Zika
B
Human anal swab
Proteinase K treatment
RPA 37 ℃ 10 min
Cas12a detection
37 ℃ 60 min
DETECTR
Collateral cleavage of reporter
C
SHERLOCK
RPA
RT-RPA
T7 transcription
Cas13a detection
Cleavage reporter
Cas13a-crRNA
Target sequence

Fig. 4

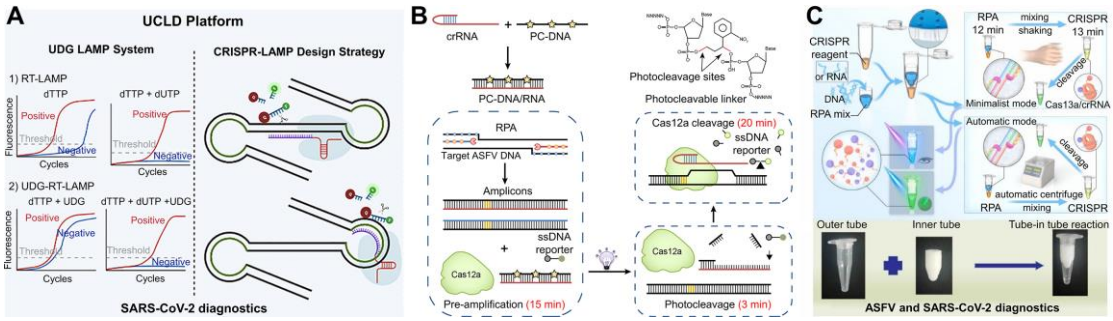

A
UCLD Platform
UDG LAMP System
CRISPR-LAMP Design Strategy
1) RT-LAMP
dTTP
dTTP + dUTP
Positive
Negative
Threshold
Fluorescence
Cycles
2) UDG-RT-LAMP
dTTP + UDG
dTTP + dUTP +UDG
SARS-CoV-2 diagnostics
B
crRNA
PC-DNA
PC-DNA/RNA
RPA
Target ASFV DNA
Amplicons
ssDNA reporter
Cas12a
Pre-amplification (15 min)
Photocleavage sites
Photocleavable linker
Cas12a cleavage (20 min)
Photocleavage (3 min)
C
CRISPR reagent
or RNA
DNA
RPA mix
RPA 12 min
mixing shaking
CRISPR 13 min
Minimalist mode
Cas13a/crRNA
Cleavage
Automatic mode
automatic centrifuge
Outer tube
Inner tube
Tube-in tube reaction
ASFV and SARS-CoV-2 diagnostics

Fig. 5

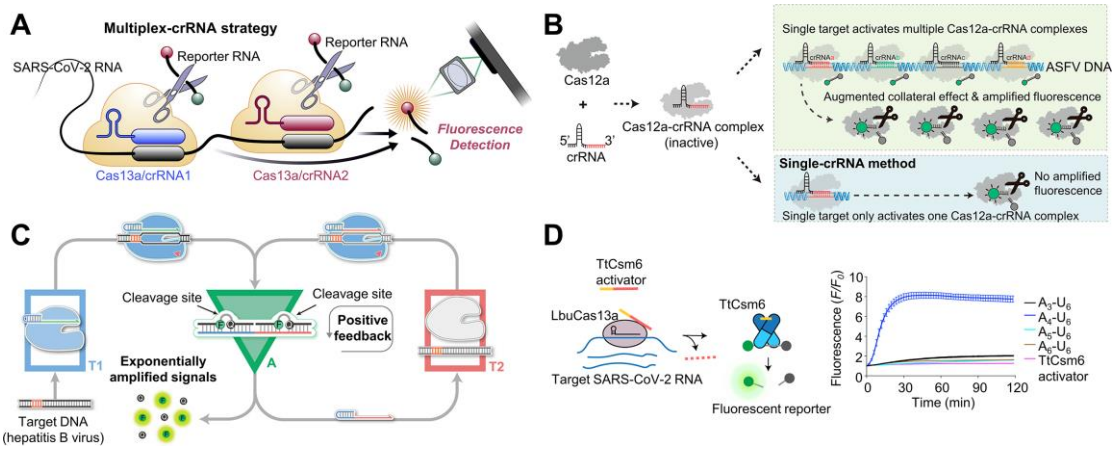

A
Multiplex-crRNA strategy
SARS-CoV-2 RNA
Reporter RNA
Reporter RNA
Fluorescence Detection
Cas13a/crRNA1
Cas13a/crRNA2
B
Cas12a
Cas12a-crRNA complex (inactive)
5'
3'
crRNA
Single target activates multiple Cas12a-crRNA complexes
ASFV DNA
Augmented collateral effect & amplified fluorescence
Single-crRNA method
No amplified fluorescence
Single target only activates one Cas12a-crRNA complex
C
Cleavage site
Cleavage site
Positive feedback
T1
T2
A
Exponentially amplified signals
Target DNA (hepatitis B virus)
D
TtCsm6 activator
LbuCas13a
TtCsm6
Target SARS-CoV-2 RNA
Fluorescent reporter
Fluorescence (F/F0)
Time (min)
A3-U6
A4-U6
A5-U6
A6-U6
TtCsm6 activator

Fig. 6

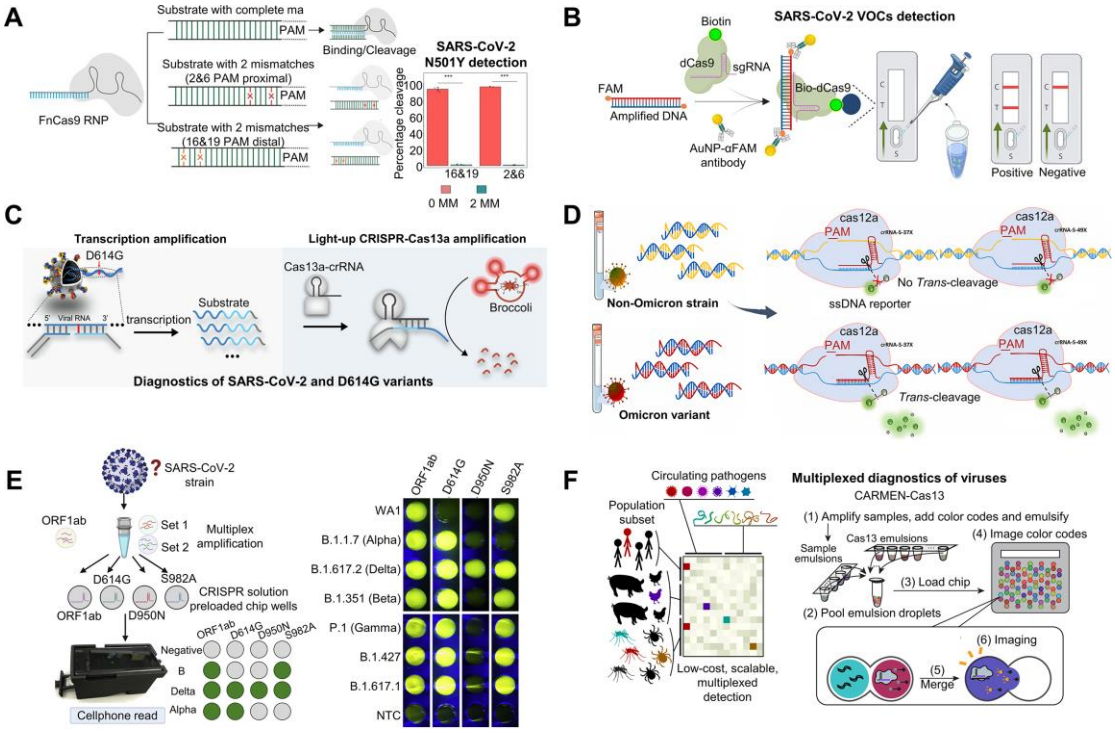

A
Substrate with complete ma
PAM
Binding/Cleavage
Substrate with 2 mismatches
(2&6 PAM proximal)
PAM
Substrate with 2 mismatches
(16&19 PAM distal)
PAM
FnCas9 RNP
SARS-CoV-2
N501Y detection
Percentage cleavage
100
80
60
40
20
0
16&19
2&6
0 MM
2 MM
B
SARS-CoV-2 VOCs detection
Biotin
dCas9
sgRNA
FAM
Amplified DNA
Bio-dCas9
AuNP-αFAM
antibody
Positive
Negative
C
Transcription amplification
Light-up CRISPR-Cas13a amplification
D614G
5'
Viral RNA
3'
transcription
Substrate
Cas13a-crRNA
Broccoli
Diagnostics of SARS-CoV-2 and D614G variants
D
cas12a
PAM
Non-Omicron strain
ssDNA reporter
No Trans-cleavage
Omicron variant
Trans-cleavage
E
SARS-CoV-2
strain
ORF1ab
Set 1
Set 2
Multiplex
amplification
D614G
S982A
ORF1ab
D950N
CRISPR solution
preloaded chip wells
Negative
B
Delta
Alpha
Cellphone read
ORF1ab
D614G
D950N
S982A
WA1
B.1.1.7 (Alpha)
B.1.617.2 (Delta)
B.1.351 (Beta)
P.1 (Gamma)
B.1.427
B.1.617.1
NTC
F
Circulating pathogens
Population
subset
Low-cost, scalable,
multiplexed
detection
Multiplexed diagnostics of viruses
CARMEN-Cas13
(1) Amplify samples, add color codes and emulsify
Sample
emulsions
Cas13 emulsions
(4) Image color codes
(3) Load chip
(2) Pool emulsion droplets
(6) Imaging
(5)
Merge

Fig. 7

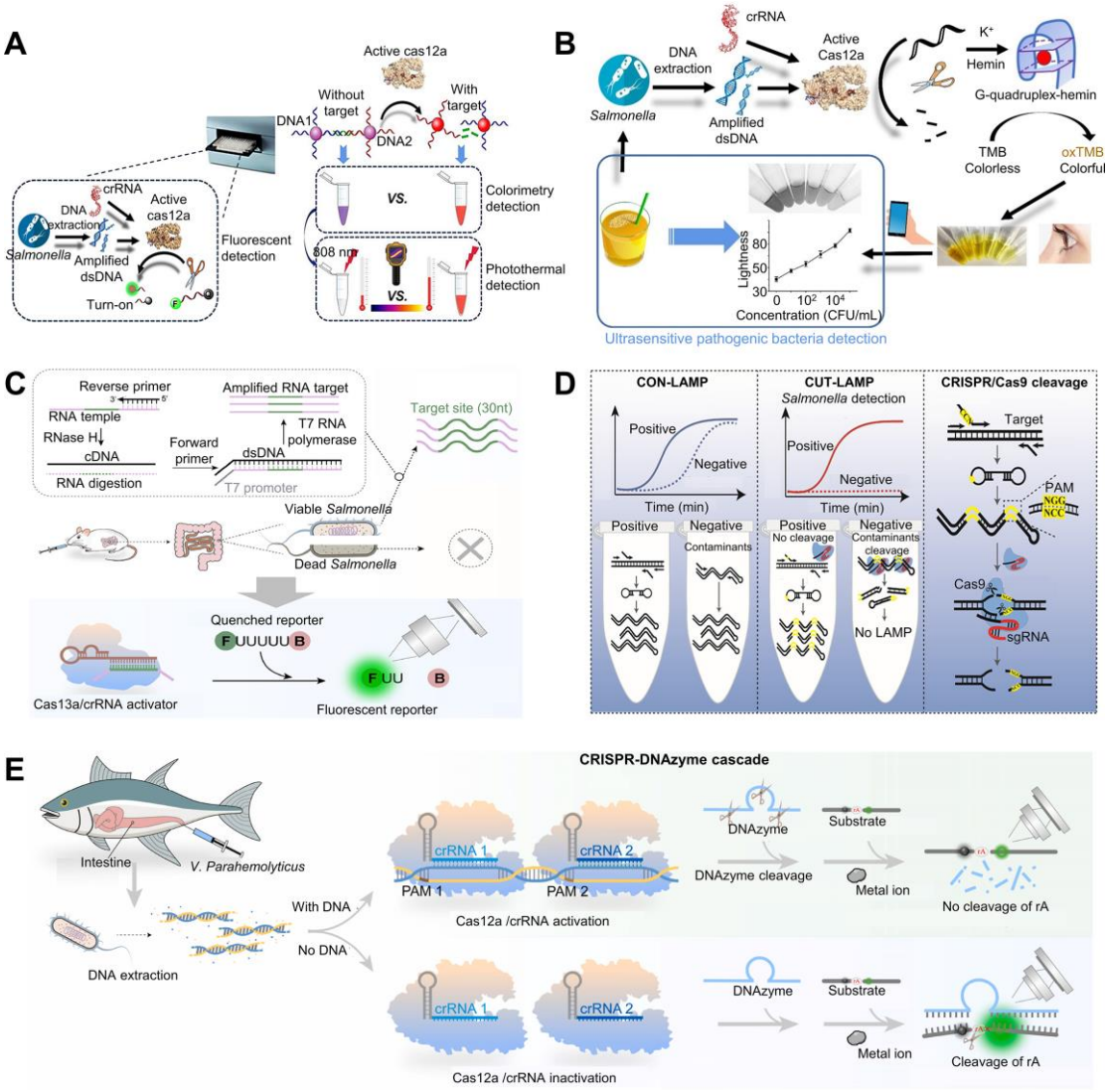

A
Active cas12a
Without target
With target
DNA1
DNA2
crRNA
DNA extraction
Active cas12a
Salmonella
Amplified dsDNA
Turn-on
Fluorescent detection
VS.
Colorimetry detection
808 nm
Photothermal detection
B
crRNA
DNA extraction
Active Cas12a
Salmonella
Amplified dsDNA
K+
Hemin
G-quadruplex-hemin
TMB Colorless
oxTMB Colorful
Lightness
Concentration (CFU/mL)
Ultrasensitive pathogenic bacteria detection
C
Reverse primer
RNA temple
RNase H
cDNA
RNA digestion
Forward primer
Amplified RNA target
T7 RNA polymerase
dsDNA
T7 promoter
Target site (30nt)
Viable Salmonella
Dead Salmonella
Quenched reporter
Cas13a/crRNA activator
Fluorescent reporter
D
CON-LAMP
CUT-LAMP
Salmonella detection
CRISPR/Cas9 cleavage
Positive
Negative
Time (min)
Contaminants
No cleavage
Contaminants cleavage
No LAMP
Target
PAM
Cas9
sgRNA
E
CRISPR-DNAzyme cascade
Intestine
V. Parahemolyticus
DNA extraction
With DNA
No DNA
crRNA 1
crRNA 2
PAM 1
PAM 2
Cas12a /crRNA activation
DNAzyme
Substrate
DNAzyme cleavage
Metal ion
No cleavage of rA
Cas12a /crRNA inactivation
Cleavage of rA

Fig. 8

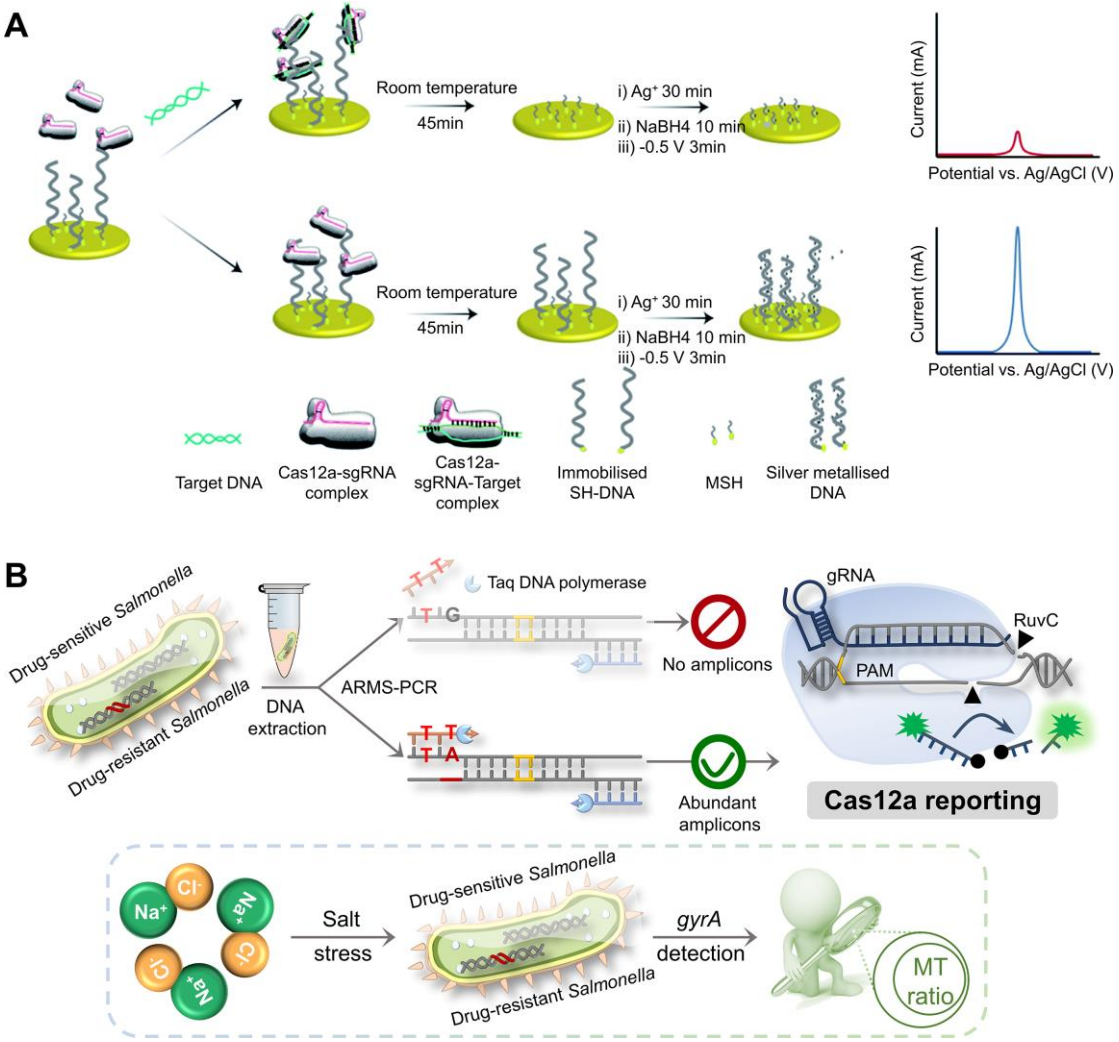
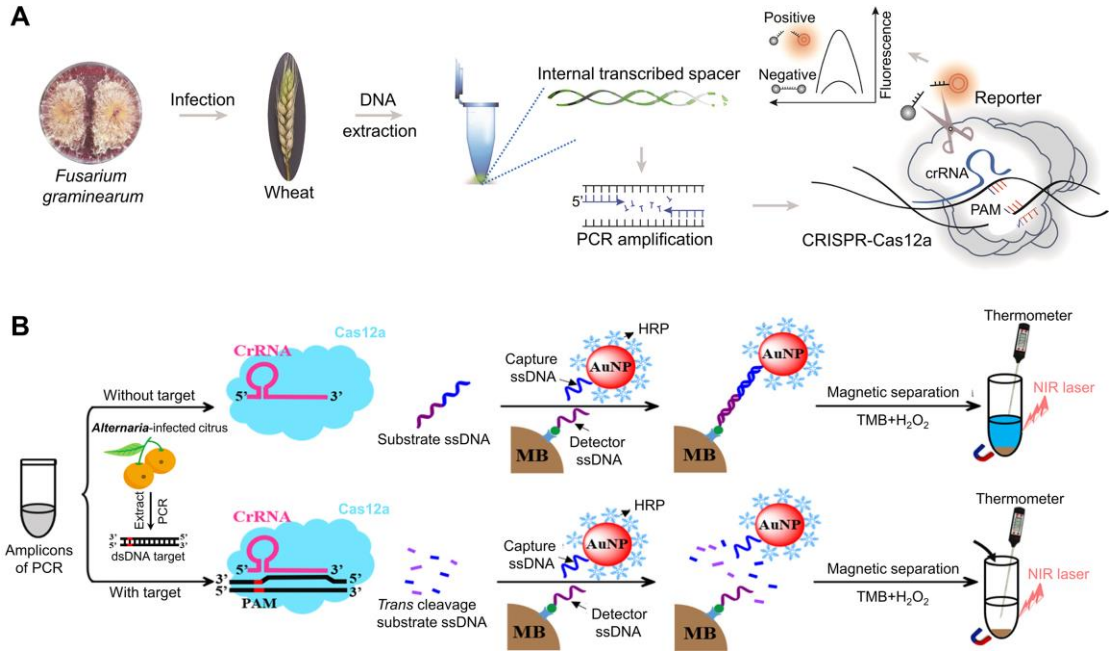

A
Room temperature
45min
i) Ag+ 30 min
ii) NaBH4 10 min
iii) -0.5 V 3min
Current (mA)
Potential vs. Ag/AgCl (V)
Target DNA
Cas12a-sgRNA complex
Cas12a-sgRNA-Target complex
Immobilised SH-DNA
MSH
Silver metallised DNA
B
Drug-sensitive Salmonella
Drug-resistant Salmonella
DNA extraction
ARMS-PCR
Taq DNA polymerase
No amplicons
Abundant amplicons
gRNA
RuvC
PAM
Cas12a reporting
Salt stress
gyrA detection
MT ratio

Fig. 9


A
Fusarium graminearum
Infection
Wheat
DNA extraction
Internal transcribed spacer
PCR amplification
Positive
Negative
Fluorescence
Reporter
crRNA
PAM
CRISPR-Cas12a
B
Amplicons of PCR
Without target
Alternaria-infected citrus
Extract
PCR
dsDNA target
With target
CrRNA
Cas12a
PAM
Substrate ssDNA
Trans cleavage substrate ssDNA
Capture ssDNA
HRP
AuNP
Detector ssDNA
MB
Magnetic separation
TMB+H2O2
Thermometer
NIR laser