Biosensors and Bioelectronics

CRISPR-based biosensors for pathogenic biosafety

--Manuscript Draft--

Manuscript Number:	BIOSBE-D-22-03857R1
Article Type:	Review Article
Section/Category:	Catalytic (enzyme, biomimetic catalysts
Keywords:	point-of-care; SARS-CoV-2 variants; drug-resistance; fungi; parasites; viable bacteria
Corresponding Author:	Ruijie Deng, Ph. D. Sichuan University Chengdu, CHINA
First Author:	Hao Yang
Order of Authors:	Hao Yang
	Rodrigo Ledesma-Amaro
	Hong Gao
	Yao Ren
	Ruijie Deng, Ph. D.
Abstract:	Pathogenic biosafety is a worldwide concern. Tools for analyzing pathogenic biosafety, that are precise, rapid and field-deployable, are highly demanded. Recently developed biotechnological tools, especially those utilizing CRISPR/Cas systems which can couple with nanotechnologies, have enormous potential to achieve point-of-care (POC) testing for pathogen infection. In this review, we first introduce the working principle of class II CRISPR/Cas system for detecting nucleic acid and non-nucleic acid biomarkers, and highlight the molecular assays that leverage CRISPR technologies for POC detection. We summarize the application of CRISPR tools in detecting pathogens, including pathogenic bacteria, viruses, fungi and parasites and their variants, and highlight the profiling of pathogens' genotypes or phenotypes, such as the viability, and drug-resistance. In addition, we discuss the challenges and opportunities of CRISPR-based biosensors in pathogenic biosafety analysis.
Suggested Reviewers:	
Opposed Reviewers:	

Prof. Dr. Ruijie Deng

College of Biomass Science and Engineering, Sichuan University

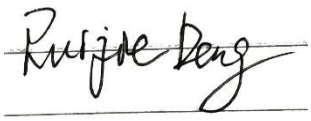
Tel: 86-028-85467382; Email: drj17@scu.edu.cn

Dec. 30th, 2022

Dear Prof. Can Dincer,

Thank you very much for your time and efforts on reviewing our manuscript (manuscript ID: **BIOSBE-D-22-03857**) entitled "*CRISPR-based biosensors for pathogenic biosafety*" by Hao Yang, Rodrigo Ledesma-Amaro, Hong Gao, Yao Ren, Ruijie Deng. We really appreciate the valuable comments from you and the reviewers, which have greatly helped us to strengthen our manuscript. We think that the manuscript is now much improved, and hope the revised version is suitable for publication in *Biosensors and Bioelectronics* as a Review Article. Our responses to the editor and reviewers' comments are listed as follows. The changes made in the manuscript are marked in red.

If there appears any question, please do not hesitate to contact me. We look forward to hearing from you for your decision at your earliest convenience.



Ruijie Deng, Ph.D, Professor
College of Biomass Science and Engineering, Healthy Food Evaluation Research Center
Sichuan University
Chengdu 610065, China
Email: drj17@scu.edu.cn

Response to the editor' comments

Dear editor:

Thank you for reviewing and handling our manuscript ("CRISPR-based biosensors for pathogenic biosafety", manuscript ID: BIOSBE-D-22-03857). We are greatly grateful to the reviewers for their suggestions which help us to improve the Review Article.

We checked the manuscript carefully and revised it according to the comments from the two reviewers. Revisions are showed point-by-point as follows based on the reviewers' comments, and changes are highlighted in the revised manuscript.

Besides, based on the comments from Editor's reports, we have revised the manuscript accordingly: (i) This article is expected to present a thorough and comprehensive overview of the chosen subject and is extensively referenced and comprise around 8000 words. (ii) We have provided six keywords below the Abstract to describe the contents of the paper, **e.g.**, point-of-care; SARS-CoV-2 variants; drug-resistance; fungi; parasites; viable bacteria. (iii) We have carefully checked the language of the manuscript, asked Prof. Rodrigo Ledesma-Amaro (Imperial College London, UK, one of the co-authors of the manuscript) to help us revise the manuscript. And the changes made in the manuscript are marked in red. (vi) We have added the recent (last 3 months) advances on the CRISPR-powered pathogen detection. All these methods have advanced the CRPSPR-based tools for analyzing pathogenic biosafety, and the updated advances are as follows:

In the section of the principle of CRISPR-based biosensors, we newly introduced a hybrid CRISPR/Cas that fuses Cas12a and Cas13a to a single protein, enabling to simultaneously target DNA and RNA in a single tube (*Biosens. Bioelectron.*, 2023, 219, 114819). The hybrid CRISPR/Cas eliminated the use of multi-enzymes, which can address the drawbacks in low-stability and incompatible reaction conditions. This is the first case for different Cas protein fusion and improve the general application of CRISPR for nucleic acid tests.

In the section of the strategies for POC testing, we newly introduced that the use of HRP-labeled oligonucleotides immobilized on the surface of magnetic beads serving as the reporter of Cas effectors can achieve target-triggered colorimetric signal output (*J. Am. Chem. Soc.*, 2022, 144(36), 16310-16315). The integration of CRIPSR with HRP-based colorimetric detection facilitates the POC use of CRIPSR-based diagnostics.

In the section related to bacterial biosafety, we newly introduced the recent one-pot RPA-Cas12a assays for POC diagnosis of *S. typhimurium* (*Biosens. Bioelectron.*, 2022, 207, 114167), *S. aureus* (*Sci. Total Environ.*, 2022, 838, 156048) and *Enterocytozoon hepatopenaei* (*Sens.*

Actuator B-Chem., 2023, 374, 132853); a preamplification-free POC strategy for *E. coli* (*Sens. Actuator B-Chem.*, 2023, 376, 133005); a colorimetric assay using Ag^{+}-TMB reporter for *S. aureus* (*Anal. Chim. Acta*, 2022, 1230, 340357) and a ratiometric fluorescent biosensing platform for ultrasensitive detection of *Salmonella typhimurium* via CRISPR/Cas12a and silver nanoclusters (*J. Hazard. Mater.*, 2023, 443, 130234). For viable bacteria diagnosis, a polyethyleneimine-encapsulated CRISPR/Cas12a-circular reporter nanoprobes (CasCLR) tool was recently reported for rapid and direct *in vivo* visualization of genomic information in living bacteria, and was presented in the revised manuscript (*Biosens. Bioelectron.*, 2022, 216, 114641). For the detection of drug-resistant bacteria, a magnetic relaxation switching strategy (C-MRS) based on Cas12a was demonstrated for the detection of methicillin-resistant *S. aureus* (MRSA) in food (*Biosens. Bioelectron.*, 2023, 222, 114984). These works show the promise of CRISPR-based biosensors for profiling the key genotypes and phenotypes of pathogenic bacteria besides taxonomy.

In viral biosafety, we newly introduced two multiplexing assays for POC detection of ASFV (*Anal. Chem.*, 2022, 94(30), 10805-10812 and *ACS Sens.*, 2022, 7(12), 3940-3946); a dual-gene simultaneous recognition strategy for POC detection of SARS-CoV-2 (*Anal. Chem.*, 2022, 94(27), 9603-9609); and two POC diagnostics for variants of concerns of SARS-CoV-2 (*Anal. Chem.*, 2022, 94(44), 15472-15480 and *Biosens. Bioelectron.*, 2022, 195, 113646). COVID-19 is a global serious issue, these assays using CIRPSR show the promise for POC test of SARS-CoV-2 infection.

In fungal biosafety, we newly introduced a photothermal detection of *Alternaria* species using HRP-TMB reporter, the colorimetric output enabled POC testing (*Int. J. Biol. Macromol.*, 2022, 222, 2661-2669). The assay involved only a thermometer, eliminating the need for cumbersome and expensive instruments traditionally used in morphological analysis and gene-sequencing.

In parasitic biosafety, we newly introduced a G-quadruplex-substrated RPA-Cas12a assay for label-free detection of *Leishmania donovani* with single-cell sensitivity (*ACS Sens.*, 2022, 7(10), 2968-2977).

Collectively, we review the advances of CRISPR biosensing for bacteria and viruses with the information of phenotypes and genotypes (such as the viability and drug-resistance) and viral variants, as well as the neglectful pathogens including fungi and parasites. These aspects have not been well reviewed before. The current published review papers on CRISPR/Cas biosensing technology, such as biosensing technology (*Trends Biotechnol.*, 2019, 37 (7), 730), point-of-care diagnosis (*Angew. Chem. Int. Ed.*, 2020, 59 (47), 20754), non-nucleic acid detection (*Biosens.*

Bioelectron., 2022, 215, 114559) and virus detection (*Biosens. Bioelectron.*, 2021, 193, 113541). *Trends Biotechnol.*, 2019, 37 (7), 730 envisioned the potential of CRISPR/Cas systems to become promising candidates for next-generation diagnostic biosensing platforms. *Angew. Chem. Int. Ed.*, 2020, 59 (47), 20754 reviewed the CRISPR-mediated biosensing toward understanding cellular biology and point-of-care diagnosis. *Biosens. Bioelectron.*, 2021, 193, 113541 summarized the advances of CRISPR-Cas based virus detection reported before 2021. *Biosens. Bioelectron.*, 2022, 215, 114559 introduced the CRISPR toolboxes for detection of non-nucleic acid targets. *Trends Biotechnol.*, 2022, 10.1016/j.tibtech.2022.11.002 compared the CRISPR/Cas and Argonaute biosensors for nucleic acid tests. These reviews have provided us a view about the advance of CRIPSR-based biosensing, but a review about the recent advances of their application for pathogenic biosafety is still highly demanded due to the field is evolving very fast. And we added the review articles in the revised manuscript.

In addition, we have carefully checked the language of the manuscript, and revised grammar errors. We think that the manuscript is now much improved, and hope the revised version is suitable for the publication in *Biosensors and Bioelectronics* as a Review Article.

Response to the Reviewers' comments

Reviewer #1:

Comment: This manuscript reviewed the CRISPR/cas biosensing technology for pathogenic biosafety. There are several review papers on CRISPR/cas biosensing technology (Trends in biotechnology, 2019, 37 (7), 730; Angewandte Chemie International Edition, 2020, 59 (47), 20754; Biosensors and Bioelectronics, 2021 193, 113541, et al.) have been published. The novelty of this manuscript is not clear. Authors are required to clearly state why this review manuscript is necessary to be published since there are so many papers published. There are lots of grammar errors.

Question 1: What is the difference between this manuscript and the published reviewers.

Response: We thank the respected review' comment associated with the advances and new ideas in the manuscript. Compared to current review papers, our manuscript mainly differs in the following features or contents:

(i) We review the advances of CRISPR biosensing for bacteria and viruses with the information of phenotypes and genotypes, as well as the neglectful pathogens including fungi and parasites. Given that the importance of pathogen's phenotypes and genotypes, such as the viability, drug-resistance, pathogenicity and infectivity of pathogens involved in biosafety, we reviewed CRIPSR-based biosensors for detecting bacteria cell, viable bacteria and drug-resistant bacteria. Detection of viable bacteria mainly relies on the RNA sensing. Identification of drug-resistant bacteria include plasmid-mediated drug-resistant ones and SNP-involved ones. The mutations in viruses can change viral pathogenicity and infectivity. We highlight the advances for detecting their mutant such as the SARS-CoV-2 variants of concern. These achievements for detecting pathogen's phenotypes and genotypes are contributed from CRIPSR-enabled precise nucleic acid tests, that even with single-nucleotide resolution. The published reviewers have not highlighted these features of CRISPR-based biosensors yet, but the CRISPR-based tools for detecting pathogen's phenotypes and genotypes have been rapidly developed, and need to be reviewed to arouse interest from molecular biology and epidemiology. Besides, pathogens including fungi and parasites are often ignored, but hold great threat to human health. CRISPR-based biosensors have showed the promise for diagnosis of the infection of fungi and parasites, particularly POC use, thus these sections will arouse interest from the researchers majoring in fungi and parasites.

(ii) In the methodological progress, we focused on the molecular recognition mode: nucleic acid biomarkers and non-nucleic acid biomarkers. Nucleic acid recognition only involved DNA and RNA sensing, while non-nucleic acid recognition requires applicable signal transduction tools such as aptamers, DNAzymes and other molecular translators. The principle by coupling other recognition affinities with CRISPR will broaden its target scope.

(iii) We highlighted and summarized the strategies for POC testing of pathogens. The signal readouts include fluorometry, colorimetry and lateral flow immunochromatography, and the visual measurement can be achieved using lateral-flow strips and mobile phones.

(vi) Given the field is evolving very fast, we try to check and update the recent (last 3 months) advances on the CRISPR-powered pathogen detection. All these methods have advanced the CRPSPR-based tools for analyzing pathogenic biosafety, the updated advances are as follows:

In the section of the principle of CRISPR-based biosensors, we newly introduced a hybrid CRISPR/Cas that fuses Cas12a and Cas13a to a single protein enables to simultaneously target DNA and RNA in a single tube (*Biosens. Bioelectron.,* 2023, 219, 114819). The hybrid CRISPR/Cas eliminated the use of multi-enzymes, which can address the drawbacks in low-stability and incompatible reaction conditions. This is the first case for different Cas protein fusion and improve the general application of CRISPR for nucleic acid tests.

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The current published review papers on CRISPR/Cas biosensing technology, such as biosensing technology (*Trends Biotechnol.*, 2019, 37 (7), 730), point-of-care diagnosis (*Angew. Chem. Int. Ed.*, 2020, 59 (47), 20754), non-nucleic acid detection (*Biosens. Bioelectron.*, 2022, 215, 114559), virus detection (*Biosens. Bioelectron.*, 2021, 193, 113541) and foodborne pathogen detection (*Compr. Rev. Food Sci. Food Saf.*, 2022, 21, 3010-3029). *Trends Biotechnol.*, 2019, 37 (7), 730 envisioned the potential of CRISPR/Cas systems to become promising candidates for next-generation diagnostic biosensing platforms. *Angew. Chem. Int. Ed.*, 2020, 59 (47), 20754 reviewed the CRISPR-mediated biosensing toward understanding cellular biology and point-of-care diagnosis. *Biosens. Bioelectron.*, 2021, 193, 113541 summarized the advances of CRISPR-Cas based virus detection in 2021. *Biosens. Bioelectron.*, 2022, 215, 114559 introduced the CRISPR toolboxes for detection of non-nucleic acid targets. *Compr. Rev. Food Sci. Food Saf.*, 2022, 21, 3010-3029 discussed the CRISPR/Cas-based detection of foodborne pathogens. *Trends Biotechnol.*, 2022, 10.1016/j.tibtech.2022.11.002 compared the CRISPR/Cas and Argonaute biosensors for nucleic acid tests. These reviews have provided us a view about

the advance of CRIPSR-based biosensing, but a review about the recent advances of their application for pathogenic biosafety is still highly demanded due to the field is evolving very fast.

We added the review articles (*Trends Biotechnol.*, 2019, 37 (7), 730; *Angew. Chem. Int. Ed.*, 2020, 59 (47), 20754; *Biosens. Bioelectron.*, 2021 193, 113541; *Biosens. Bioelectron.*, 2022, 215, 114559, *Compr. Rev. Food Sci. Food Saf.*, 2022, 21, 3010-3029 and *Trends Biotechnol.*, 2022, 10.1016/j.tibtech.2022.11.002) in the revised manuscript.

We have carefully checked the language of the manuscript, and revised grammar errors. Besides, we asked Prof. Rodrigo Ledesma-Amaro (Imperial College London, UK, one of the co-authors of the manuscript) to help us revise the manuscript.

Question 2: What are take home message for the audience, is not clear.

Response: The Review is expected to show a comprehensive view of the recent (last 3 or 6 months) advances of CRISPR biosensing for pathogens including bacteria, viruses, fungi, parasites and their variants of concern. Given that the importance of pathogen's phenotypes and genotypes, such as the viability, drug-resistance, pathogenicity and infectivity of pathogens involved in biosafety, we also discussed the CRIPSR-based biosensors for identification of viable bacteria and drug-resistant bacteria, and highlighted the advances for diagnostics of SARS-CoV-2 variants of concern. Moreover, the current published reviews about CRISPR-based biosensing often ignored the infrequent pathogens including fungi and parasites, but which holds great threat to human health. The discussion of CRISPR-based biosensors for diagnosis of fungi and parasites thus enables to arouse interest from the researchers majoring in fungi and parasites. Furthermore, we summarized the strategies for POC testing of CRISPR-based biosensing in pathogenic diagnosis, this summary maybe arouse interest from molecular biology and epidemiology. There have several published reviews focused on CRISPR applications in pathogenic biosafety, such as biosensing technology (*Trends Biotechnol.*, 2019, 37 (7), 730), point-of-care diagnosis (*Angew. Chem. Int. Ed.*, 2020, 59 (47), 20754), non-nucleic acid detection (*Biosens. Bioelectron.*, 2022, 215, 114559), virus detection (*Biosens. Bioelectron.*, 2021, 193, 113541) and foodborne pathogen detection (*Compr. Rev. Food Sci. Food Saf.*, 2022, 21, 3010-3029). Among them, *Trends Biotechnol.*, 2019, 37 (7), 730 envisioned the potential of CRISPR/Cas systems to become promising candidates for next-generation diagnostic biosensing platforms; *Angew. Chem. Int. Ed.*, 2020, 59 (47), 20754 reviewed the CRISPR-mediated biosensing toward understanding cellular biology and point-of-care diagnosis; *Biosens. Bioelectron.*, 2021 193, 113541 summarized the advances of CRISPR/Cas based virus detection

in 2021; *Compr. Rev. Food Sci. Food Saf.*, 2022, 21, 3010-3029 discussed the CRISPR/Cas-based detection of foodborne pathogens; *Trends Biotechnol.*, 2022, 10.1016/j.tibtech.2022.11.002 compared the CRISPR/Cas and Argonaute biosensors for nucleic acid tests. These reviews have provided us a view about the advance of CRIPSR-based biosensing, while some of which partly cover the pathogen biosensing, mainly such as bacteria and viruses, they lack the content reviewing CRISPR tools for biosensing phenotypes and genotypes of bacteria, and fungi, parasites as well as pathogenic variants of concern.

Question 3: In addition, in the section 2: principle of CRISPR/Cas. It is classified as nucleic acid based and non-nucleic acid based. But the 2.3 POC detection should not belong to the principle of CRISPR/Cas. The signal readout formats need to summarized.

Response: Thank you for your advice, we have adjusted the POC detection to an independent section, *i.e.*, Section 3 (**Strategies for POC testing**) in the main manuscript. The fast and cost-effective signal readouts including fluorometry, colorimetry and lateral flow immunochromatography, were preferred for POC testing, achieving visual measurement with the assistance of lateral-flow papers and mobile phones (Fig. R1, *i.e.*, Fig. 4 in the main manuscript). In addition, we newly introduced that use of HRP-labeled oligonucleotides immobilized on the surface of magnetic beads serving the reporter of Cas effectors can achieve target-triggered colorimetric signal output (Fig. R1B, *i.e.*, Fig. 4B in the main manuscript) (*J. Am. Chem. Soc.*, 2022, 144(36), 16310-16315).

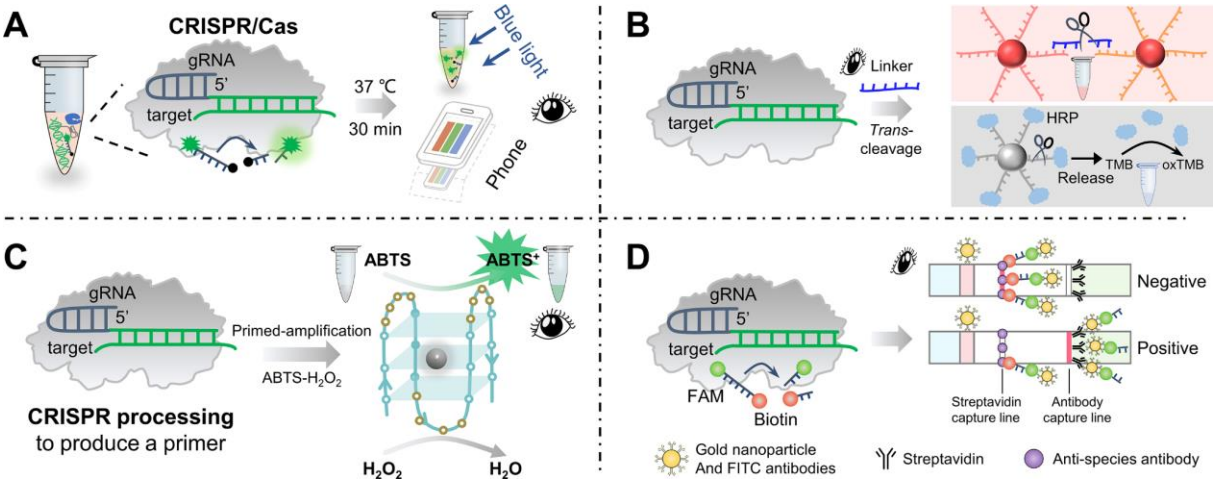


Fig. R1. Strategies for CRISPR-based POC testing. (A) CRISPR-based visual assay via fluorescence under a light excitation, the reporting signal can be observed by naked eyes or smartphones (*Cell*, 2021, 184(2), 323-333.e329; *Anal. Chem.*, 2019, 91(19), 12156-12161). (B) The CRISPR-mediated tans-cleavage towards the single-strand DNA/RNA linkers which behave as the junctions between gold nanoparticles or between

magnetic bead and HRP, resulting in a colour change in solution (*Biosens. Bioelectron.*, 2021, 172, 112749; *J. Am. Chem. Soc.*, 2022, 144(36), 16310-16315). (C) CRISPR processing produces a primer to initiate the subsequent amplification, leading to produce abundant G4/hemin complexes that can catalyze the conversion of colorless ABTS-H_2O_2 into green (*Talanta*, 2021, 233, 122554). (D) Lateral-flow assays (LFAs) contribute a simple visualization via taking advantage of reporters carrying fluorescein and biotin bind to anti-fluorescein isothiocyanate (FITC) antibodies coupled to gold nanoparticles in the sample pad of the strip (*Science*, 2021, 360(6387), 444).

Question 4: Challenges and perspectives associated with CRISPR/cas based pathogenic biosafety monitoring need to be specified in details.

Response: Thank you for your critical advice. We have tried to specify the challenges and perspectives in details, mainly considered (i) pathogenic diagnosis by CRISPR biosensing in complex matrix requires rapid and convenient extraction processes; (ii) pathogenic variants with gene mutations highlight the demand for assays capable of SNP discrimination; (iii) preamplification makes the detection process complicated, time-consuming, and possibly induces false-positive caused by contamination; (iv) gaining precise and conclusive information by genomic diagnosis requires detection of several target sequences simultaneously, so-called multiplexing detection. The improved content in the main manuscript (Page 45-46, Line 650-672) shows as follows:

Several challenges remain in applying CRISPR-based biosensors for practical and commercialized use. First, in field diagnostics, the complex matrix derived from samples could inference the amplification and signal yield, so pretreatments such as extraction and purification must be considered. Development of rapid and convenient extraction devices, such as microneedles (*ACS nano*, 2019, 13(6), 6540-6549) and microfiber filters (*ACS Synth. Biol.*, 2022, 11(5), 1772-1781), can be an effective path to facilitate the CRISPR-based biosensors for on-site diagnostics. Second, pathogenic variants, particularly those associated with single-nucleotide mutation, highlight the demand for assays with SNP-recognizable specificity. Reasonable chemical modification and artificial mismatches in gRNA (*J. Am. Chem. Soc.*, 2022, 144(28), 12584-12594; *Anal. Chem.*, 2022, 94(48), 16953-16959), as well as engineered Cas proteins (such as split Cas12a) (*Nat. Chem. Biol.*, 2019, 15(9), 882-888) possibly enable to improve the assaying specificity. Third, preamplification makes the detection process complicated, time-consuming, and possibly induces false-positive caused by contamination. Effective strategies could be the use of multiplex Cas effectors, cascade CRISPR assays (*Science*, 2018, 360(6387), 439; *Chem. Commun.*, 2021, 57(2), 247-250; *Sci. Adv.*, 2021, 7(5), eabc7802)

and droplet microfluidic platforms (*Nat. Commun.*, 2020, 11(1), 6202; *ACS Nano*, 2021, 15(1), 1167-1178) that not only allow nucleic acid preamplification-free detection but also sensitive quantification in the femtomolar range. Fourth, numerous pathogenic variants involve multiple mutations and infections are often caused by multiple strains, thus highlighting the development of multiplexing assays. Different targeting Cas effectors and reporting approaches should be exploited to meet the multiplex profiling, where the class 1 CRISPR systems (*Nat. Rev. Microbiol.*, 2022, 20(6), 351-364) and protein reporter-based CRISPR systems (e.g., Craspase) (*Science*, 2022, 377(6612), 1278-1285) could be focused.

Question 5: References need to be updates since some recent papers are not cited, such as Biosensors and Bioelectronics, 2022, 207, 114167

Response: The field is evolving very fast, we have tried to check and update the recent (last 3 months) advances on the CRISPR-powered pathogen detection. We emphatically focused on the reported assays capable of POC testing, among which the most outstanding works include: (i) a microfluidic paper-based Cas12a assay for on-site biosensing of *Salmonella typhimurium* (*Biosens. Bioelectron.*, 2022, 207, 114167) (Page 20, Line 348-349); (ii) a AuNPs-based Cas12 colorimetric strategy for POC diagnosis of SARS-CoV-2 using smartphone readout (*Biosens. Bioelectron.*, 2022, 195, 113646) (Page 30-31, Line 528-532); (iii) a CRISPR-Cas12a-powered dual-mode biosensor for cross-validating and visual detection of pathogenic bacteria (*ACS Sens.*, 2021, 6(8), 2920-2927) (Page 37, Line 642); (vi) a smartphone-read G-quadruplex-based Cas12a bioassay for detection of bacteria (*Sens. Actuators B.*, 2021, 347, 130586) (Page 17, Line 288-295); (v) a ratiometric fluorescent biosensing platform for ultrasensitive detection of Salmonella typhimurium via CRISPR/Cas12a and silver nanoclusters (*J. Hazard. Mater.*, 2023, 443, 130234) (Page 21, Line 353-360). Paper-based microfluidic devices have several advantages over conventional microfluidic devices including simpler fabrication, lower cost, easier disposal, and the ability to operate without pumps or other supporting equipment. Smartphones integrating camera, data analyzer, interactivity and potability poses high applicability in biosensing. Therefore, microfluidic paper and smartphone have become the most popular POC testing readouts. Other outstanding works in POC testing include: (vi) an Ag^{+}-TMB-signaling colorimetric Cas12a assay for visual detection of *Staphylococcus aureus* (*Anal. Chim. Acta*, 2022, 1230, 340357) (Page 21, Line 360-365), (vii) a urease-catalyzed hydrolytic Cas12a assay for POC detection of ASFV (*ACS Sens.*, 2022, 7(12), 3940-3946) (Page 28-29, Line 486-489), and (viii) a photothermal Cas12a platform using HRP-TMB reporter detection of *Alternaria* species (*Int. J. Biol. Macromol.*, 2022, 222, 2661-2669) (Page 34, Line 572-578).

Reviewer #2:

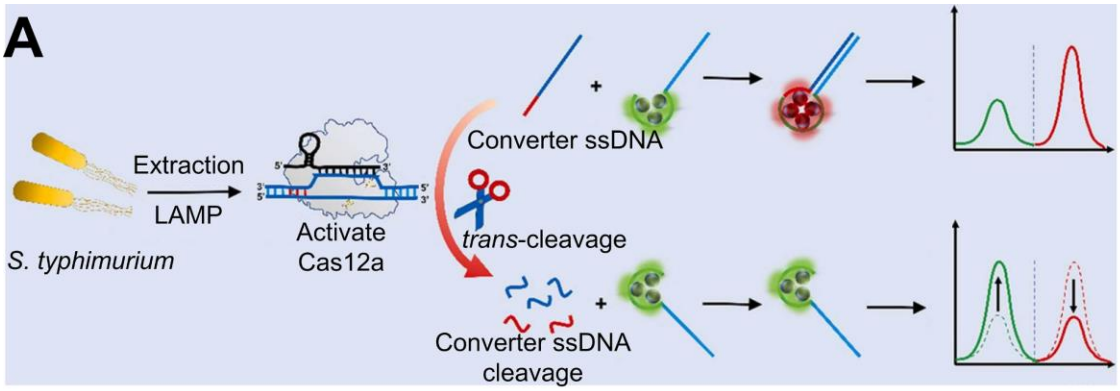
Question 1: Abstract should be refined. In current form it is very lengthy and not solving the purpose of abstract.

Response: Thank you for your reminder. The abstract has been refined. We have deleted the redundant content, *i.e.*, "The review will introduce CRISPR-based technologies and their contribution to pathogenic biosafety analysis"; and added an indispensable content (marked in red), the improved Abstract is showed as follows:

Pathogenic biosafety is a worldwide concern. Tools for analyzing pathogenic biosafety, that are precise, rapid and field-deployable are highly demanded. Recently developed biotechnological tools, especially those utilizing CRISPR/Cas systems which can couple with nanotechnologies, have enormous potential to achieve point-of-care (POC) testing for pathogen infection. In this review, we first introduce the working principle of class II CRISPR/Cas system for detecting nucleic acid and non-nucleic acid biomarkers, and highlight the molecular assays that leverage CRISPR technologies for POC detection. We summarize the application of CRISPR tools in detecting pathogens, including pathogenic bacteria, viruses, fungi and parasites and their variants, and highlight the profiling of pathogens' genotypes or phenotypes, such as viability, and drug-resistance. In addition, we discuss the challenges and opportunities of CRISPR-based biosensors in pathogenic biosafety analysis.

Question 2: Figures are usually very helpful while reading a review. Accordingly, addition of more figures will increase the importance of this paper.

Response: Deeply thank you for your advice. To increase the importance and readability of this paper, we have added 4 figures delineating the detection of bacteria (Fig. R2. *i.e.*, Fig. 5 in the manuscript), viruses (Fig. R3. *i.e.*, Fig. 6 in the manuscript), fungi (Fig. R4. *i.e.*, Fig. 7 in the manuscript) and parasites (Fig. R5. *i.e.*, Fig. 8 in the manuscript), respectively.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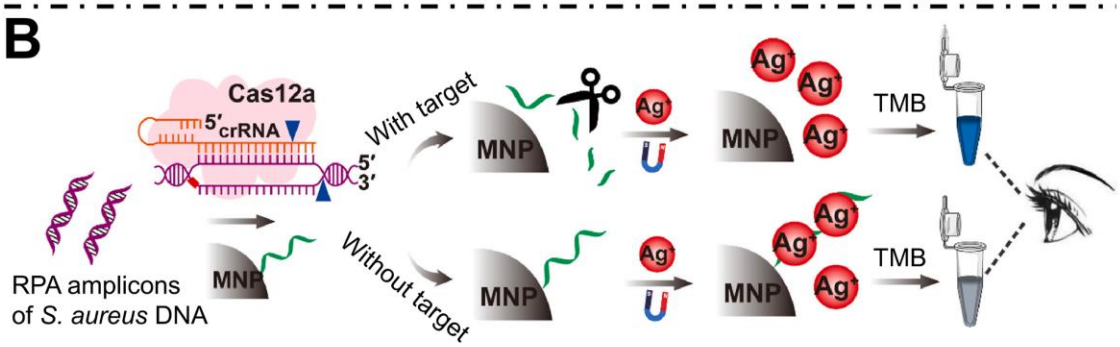
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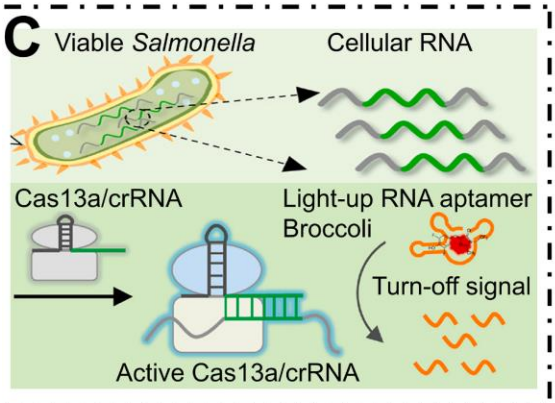
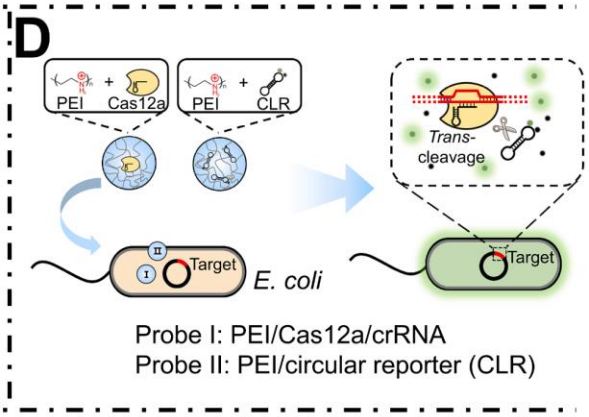
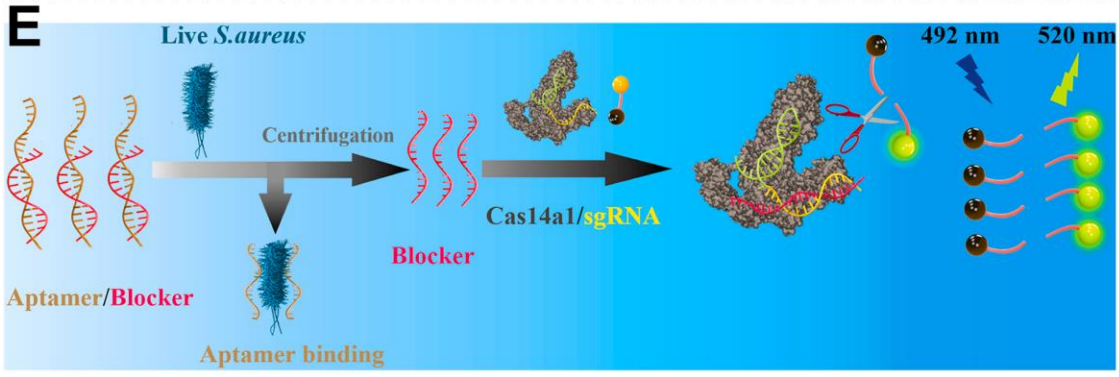

C
Viable Salmonella
Cellular RNA
Cas13a/crRNA
Light-up RNA aptamer
Broccoli
Turn-off signal
Active Cas13a/crRNA


D
PEI
Cas12a
PEI
CLR
Trans-
cleavage
Target
E. coli
Target
Probe I: PEI/Cas12a/crRNA
Probe II: PEI/circular reporter (CLR)


E
Live S.aureus
Centrifugation
Cas14a1/sgRNA
Blocker
Aptamer/Blocker
Aptamer binding
492 nm
520 nm

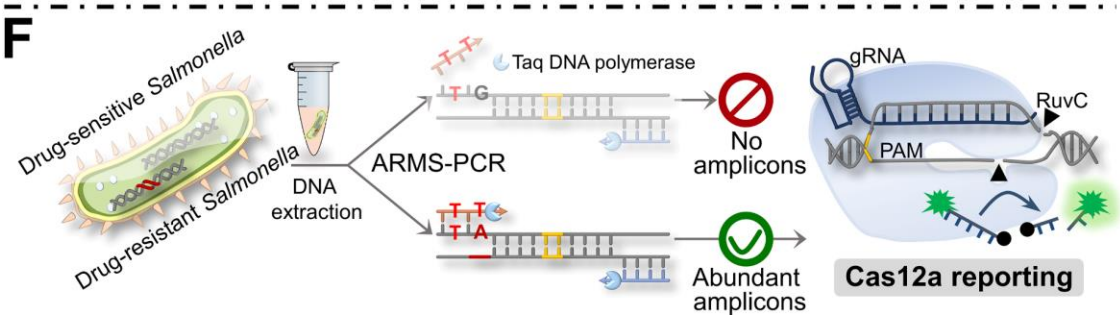

F
Drug-sensitive Salmonella
Drug-resistant Salmonella
DNA
extraction
ARMS-PCR
Taq DNA polymerase
No
amplicons
Abundant
amplicons
gRNA
RuvC
PAM
Cas12a reporting

Fig. R2. CRISPR-based biosensors for detection of pathogenic bacteria. (A) AgCNs-based Cas12a assay for ratiometric detection of *S. typhimurium* (*J. Hazard. Mater.*, 2023, 443, 130234). The dual green-to-red fluorescent outputs with self-calibration function alleviated the cause of false-positive or negative results. (B) Colorimetric Cas12a assay using Ag^+-TMB reporter for detection of *S. aureus* (*Anal. Chim. Acta*, 2022, 1230, 340357). The target-triggered color change of Ag^+-TMB reporter posed the great potential for POC application. (C) Label-free Cas13a assay using light-up RNA aptamer broccoli as the reporter for directly detection of viable *Bacillus cereus* (*Biosens. Bioelectron.*, 2021, 176, 112906). Use of RNA-targeting Cas13a evaded the application of RT. (D) In vivo visualization of genomic information in living bacteria *E. coli* by Cas12a (*Biosens. Bioelectron.*, 2022, 216, 114641). Circularization of reporter significantly improved the resistance to endonuclease in living cell. (E) An aptamer-based Cas14a platform for amplification-free detection of viable *S. aureus* (*Biosens. Bioelectron.*, 2022, 211, 114282). Use of aptamer binding the active proteins on the surface of viable bacteria cell can indicate the living cell state. (F) Cas12a signaling ARMS-PCR for detection of SNP-involved drug-resistant *S. enterica* (*J. Agric. Food Chem.*, 2022, 70(27), 8451-8457). Dual recognition and amplification via ARMS-PCR and Cas12a binding enabled this assay high specificity.

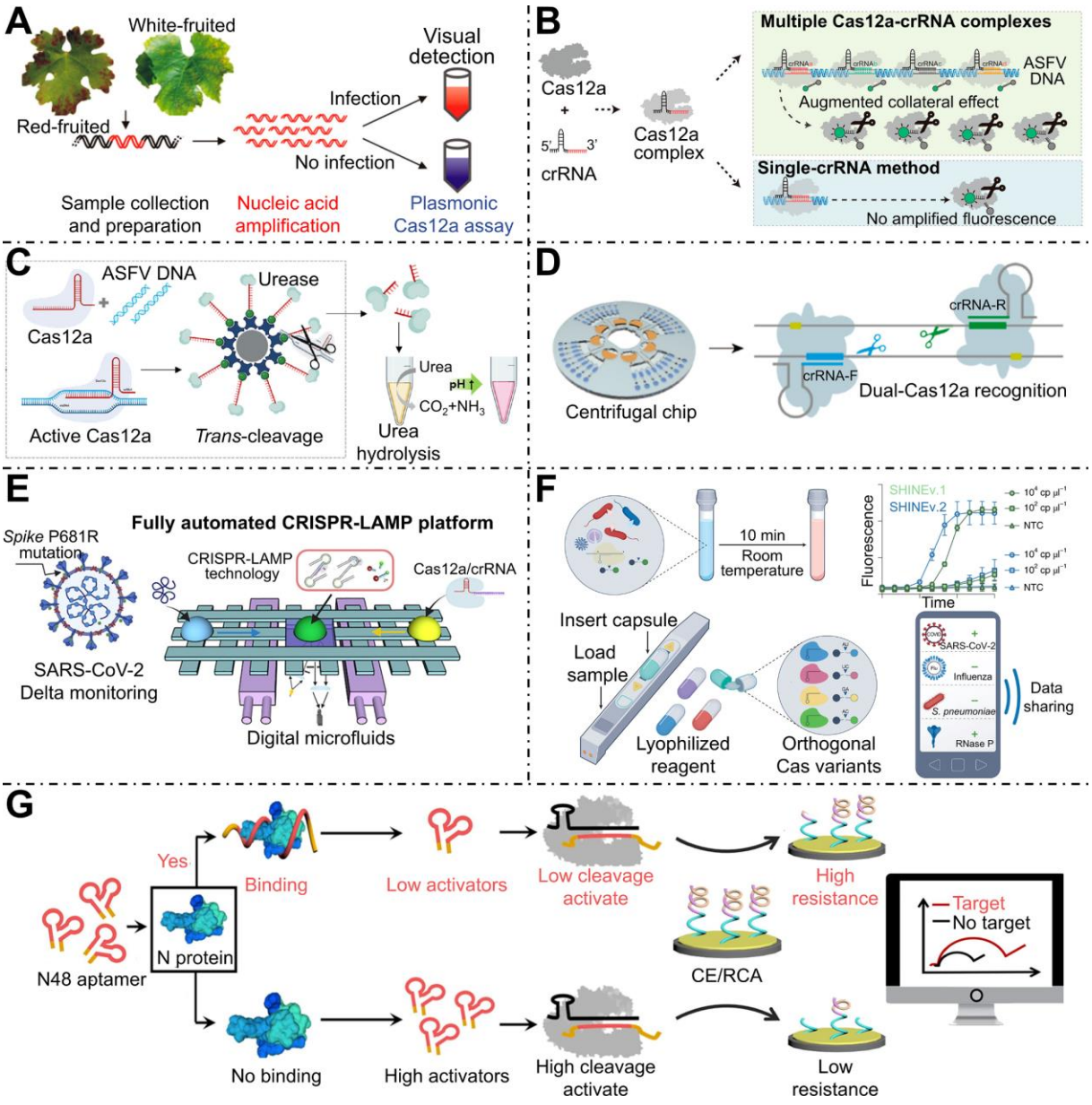
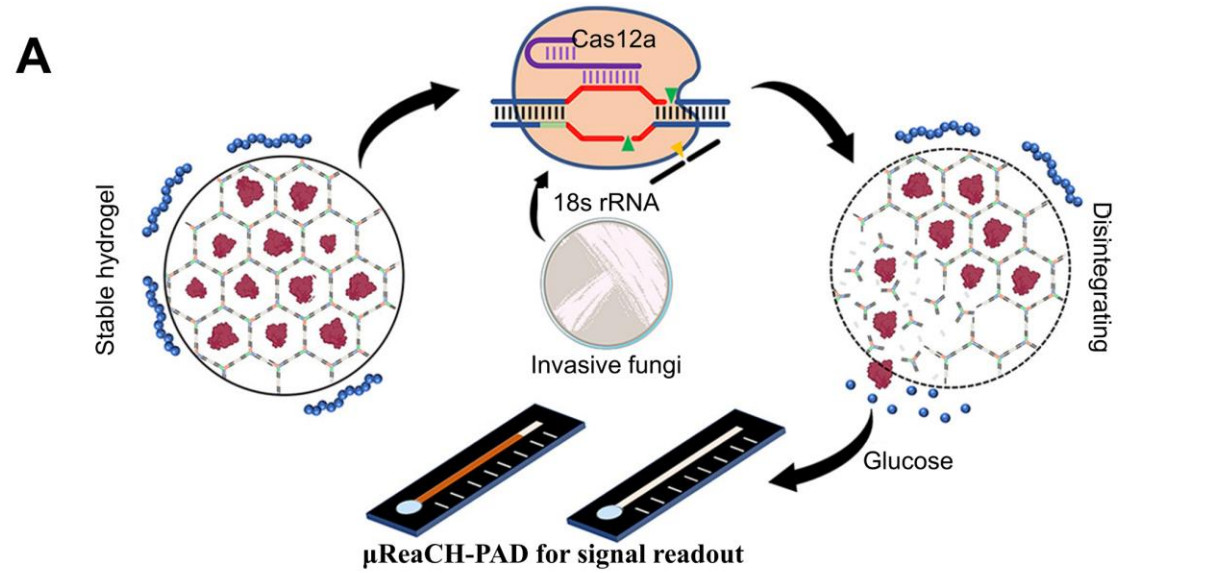
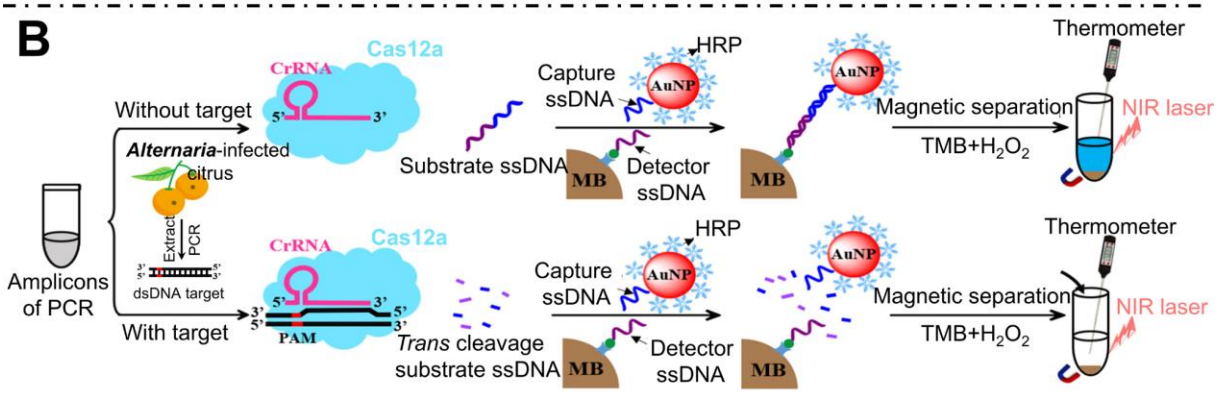
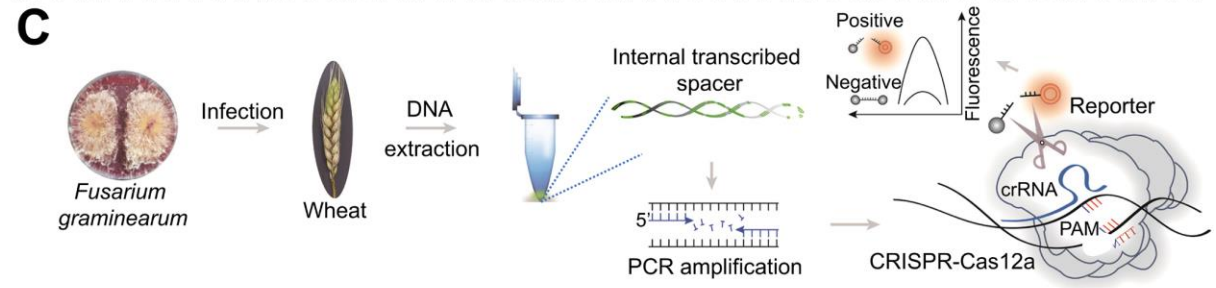


Fig. R3. CRISPR-based biosensors for detection of pathogenic virus. (A) AuNPs-based Cas12a assay for detection of GRBV (*Anal. Chem.*, 2019, 91(18), 11510-11513). Using DNA functionalized gold nanoparticles as the reporter of Cas12a cleavage enabled visual POC testing. (B) Multiple Cas12a/crRNA recognition for preamplification-free detection of ASFV (*Anal. Chem.*, 2022, 94(30), 10805-10812). Multiplexing detection showed ~64-fold stronger sensitivity than that using single crRNA for detecting ASFV. (C) Urease-catalyzed hydrolytic Cas12a assay for detection of ASFV (*ACS Sens.*, 2022, https://doi.org/10.1021/acssensors.2c02007). Using urease-catalyzed urea hydrolysis as reporter of Cas12a cleavage enabled visual POC testing. (D) Dual-Cas12a recognition for highly specific and sensitive detection of SARS-CoV-2 (*Anal. Chem.*, 2022, 94(27), 9603-9609). Design of "sample-to-answer" microfluidic enabled automatically POC sensing. (E) CRISPR-LAMP platform with artificial mismatches for detection of SARS-CoV-2 VOCs (*Anal. Chem.*, 2022, 94(44), 15472-15480). Use of digital microfluids enabled fully automated POC testing. (F) One-pot SHERLOCK

assay (SHINE) for RNA-extraction-free and isothermal POC testing of SARS-CoV-2 and its VOCs (*Nat. Biomed. Eng.*, 2022, 6(8), 932-943). SHINEv.2 leveraged lyophilised reagents and rapid sample lysis at ambient temperature making it more suitable for POC testing than SHINEv.1. (G) Aptamer-based Cas12a assay for label-free and electrochemical detection of SARS-CoV-2 particles (*Bioelectrochemistry*, 2022, 146, 108105). Use of aptamer enabled amplification-free and RNA-extraction-free sensing.


A
Cas12a
18s rRNA
Invasive fungi
Stable hydrogel
Disintegrating
Glucose
μReaCH-PAD for signal readout


B
Without target
Alternaria-infected citrus
Extract
PCR
dsDNA target
Amplicons of PCR
With target
CrRNA
Cas12a
PAM
Capture ssDNA
HRP
AuNP
Substrate ssDNA
Detector ssDNA
MB
Trans cleavage substrate ssDNA
Magnetic separation
TMB+H2O2
Thermometer
NIR laser


C
Fusarium graminearum
Infection
Wheat
DNA extraction
Internal transcribed spacer
PCR amplification
Positive
Negative
Fluorescence
Reporter
crRNA
PAM
CRISPR-Cas12a

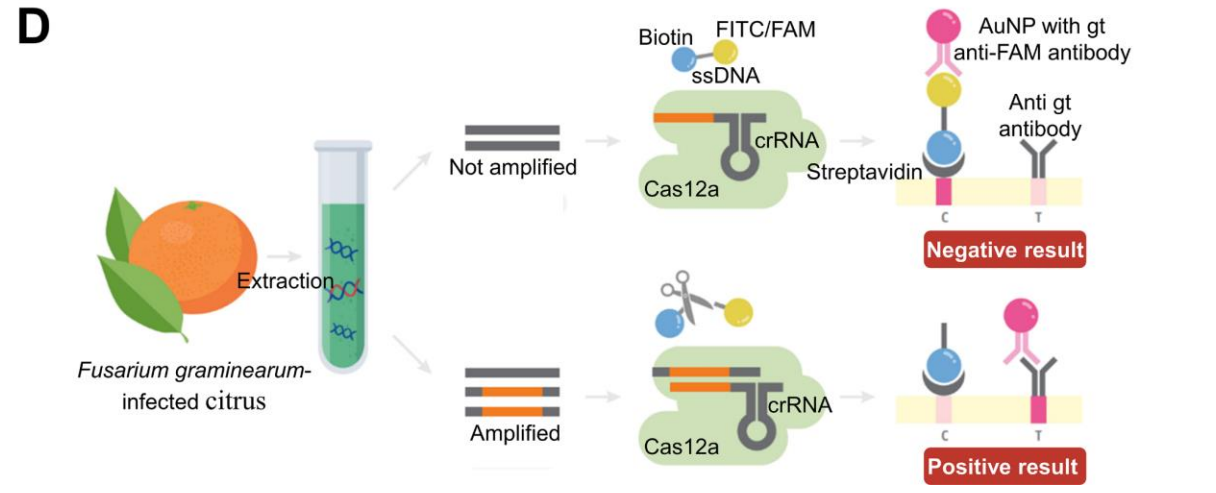

D
Extraction
Fusarium graminearum-infected citrus
Not amplified
Amplified
Biotin
FITC/FAM
ssDNA
crRNA
Cas12a
Streptavidin
AuNP with gt anti-FAM antibody
Anti gt antibody
C
T
Negative result
Positive result

Fig. R4. CRISPR-based biosensors for detection of pathogenic fungi. (A) Hydrogel paper-based Cas12a assay for detection of invasive fungi (*Candida* and *Aspergillus*) (*Anal. Chem.*, 2022, 93(50), 16965-16973). Use of microfluidic ruler-readout enabled visual POC sensing by naked eye. (B) Photothermal Cas12a platform for detection of *Alternaria* species in fruit (*Int. J. Biol. Macromol.*, 2022, 222, 2661-2669). Use of HRP-TMB reporter and thermometer enabled photothermal POC testing. (C) PCR-coupled Cas12a-based nucleic acid assay for detection of *F. graminearum* infection in wheat (*J. Agric. Food Chem.*, 2022, 70(23), 7240-7247). This assay can diagnose a 4-day *F. graminearum* infection. (D) RPA-Cas12a assay for detection of *F. graminearum* infection in citrus (*Plants*, 2021, 10(10), 2132). Use of LFA enabled POC diagnosis of citrus scab.

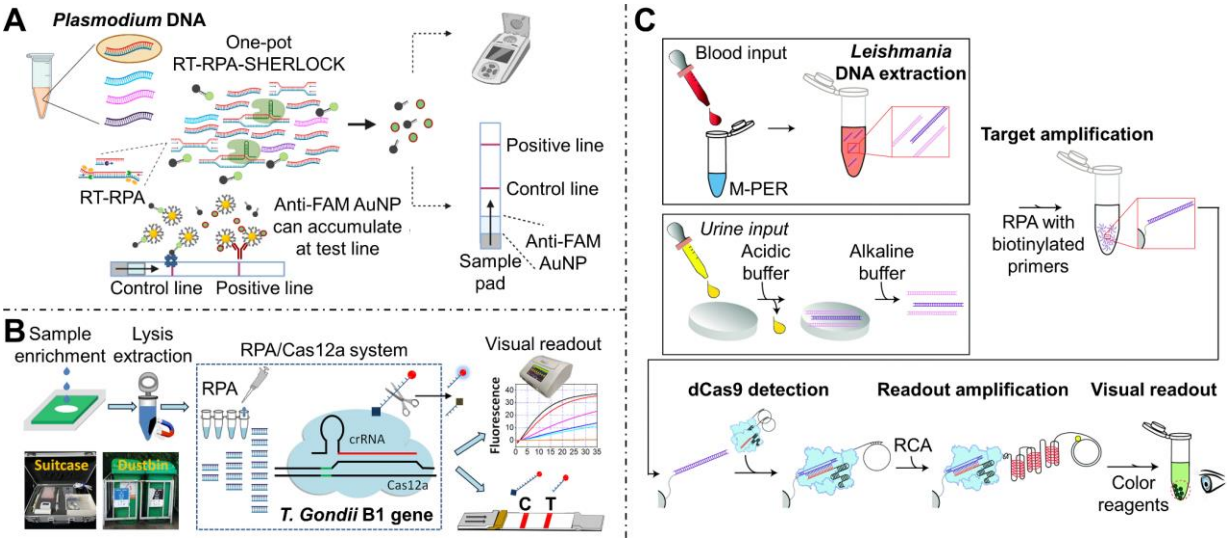


Fig. R5. CRISPR-based biosensors for detection of pathogenic parasites. (A) One-pot RT-RPA-SHERLOCK assay for detection of the malaria-causative Plasmodium species (*Proc. Natl. Acad. Sci.*, 2020, 117(41), 25722-25731). Use of LFA enabled POC diagnosis. (B) RPA-Cas12a-based nucleic acid assay to detect T. gondii (*ACS Synth. Biol.*, 2022, 11(5), 1772-1781). Use of a glass microfiber filter device for sample enrichment and lysis extraction, enabling nucleic acid extraction of low-concentration of T. gondii. (C) dCas9 coupling with RCA for detection of Leishmania with a zM-sensitivity (*Nanoscale*, 2022, 14(5), 1885-1895). Use of G-quadruplexes DNAzyme enabled a visual detection.

Question 3: English can be improved at several spots.

Response: We have attempted to improve the writing of the manuscript, asked native speaker Prof. Rodrigo Ledesma-Amaro (Imperial College London, UK) to help us revised the manuscript. And the changes made in the manuscript are marked in red.

Question 4: In the "Highlights", the words should be more concise, with refined text per sentence.

Response: We have tried to further refine the “Highlights”. The refined “Highlights” are as follows:

1. CRISPR-leveraged biosensors for POC testing of pathogenic biosafety
2. Integration of bioaffinities broadens target scope of CRISPR-based biosensing
3. CRISPR-enabled pathogenic biosafety analysis was highlighted
4. Challenges of CRISPR-based biosensing in pathogenic biosafety were discussed

Question 5: Letter in caps in the middle of the sentence, correct them. The manuscript should be thoroughly checked for these types of mistakes.

Response: Deep apology for these types of mistakes. We have tried to check and correct the mistakes throughout the manuscript.

Question 6: Second paragraph in the "Introduction" section needs some more clarity. Please fix it with more clarity and more adequate references.

Response: We have tried to clarify the second paragraph in the “Introduction” section, and cite several adequate references describing the discovery of various CRISPR systems and their first application in detection of pathogens. We have further enriched the content of CRISPR-based molecular detection, strategies for POCT diagnosis and the biosensing applications. And the changes made in the manuscript are marked in red. The improved content is as follows:

In this article, we first reviewed the mechanism and properties of class II CRISPR/Cas system, and discussed how CRISPR tools (Cas9, Cas12 and Cas13) have been leveraged for molecular assays for detecting nucleic acid and non-nucleic acid biomarkers (Fig. 1). (*J. Bacteriol.*, 1987, 169(12), 5429-5433; *Sci. Rep.*, 2014, 4(1), 6420; *Science*, 2017, 356(6336), 438; *Science*, 2018, 360(6387), 436; *Science*, 2018, 360(6387), 439; *Science*, 2018, 362(6416), 839-842; *Proc. Natl. Acad. Sci.*, 2020, 117(41), 25722-25731). We then highlighted the strategies for CRISPR-based visual biosensing that capable of POCT diagnosis, such as the readouts using lateral-flow assay (LFA) papers and smartphones. We next summarized the CRISPR-based biosensing applications in pathogenic biosafety analysis including bacteria, viruses, fungi, parasites and their variants, in which we further highlighted the analysis of pathogens’ genotypes or phenotypes, such as viability, and drug-resistance. Finally, challenges and opportunities of the CRISPR-based biotechnologies for pathogenic biosafety are also discussed.

1. CRISPR-leveraged biosensors for POC testing of pathogenic biosafety
2. Integration of bioaffinities broadens target scope of CRISPR-based biosensing
3. CRISPR-enabled pathogenic biosafety analysis was highlighted
4. Challenges of CRISPR-based biosensing in pathogenic biosafety were discussed

The authors declared that they have no conflicts of interest to this work.

CRISPR-based biosensors for pathogenic biosafety

Hao Yang[1], Rodrigo Ledesma-Amaro[2], Hong Gao[1], Yao Ren[1,*], Ruijie Deng[1,*]

[1] College of Biomass Science and Engineering, Healthy Food Evaluation Research Center, Sichuan University, Chengdu 610065, China

[2] Department of Bioengineering, Imperial College Centre for Synthetic Biology, Imperial College London, London SW7 2AZ, UK

Corresponding author

Ruijie Deng, e-mail: reny@scu.edu.cn; drj17@scu.edu.cn

Abstract:

Pathogenic biosafety is a worldwide concern. Tools for analyzing pathogenic biosafety, that are precise, rapid and field-deployable, are highly demanded. Recently developed biotechnological tools, especially those utilizing CRISPR/Cas systems which can couple with nanotechnologies, have enormous potential to achieve point-of-care (POC) testing for pathogen infection. In this review, we first introduce the working principle of class II CRISPR/Cas system for detecting nucleic acid and non-nucleic acid biomarkers, and highlight the molecular assays that leverage CRISPR technologies for POC detection. We summarize the application of CRISPR tools in detecting pathogens, including pathogenic bacteria, viruses, fungi and parasites and their variants, and highlight the profiling of pathogens' genotypes or phenotypes, such as the viability, and drug-resistance. In addition, we discuss the challenges and opportunities of CRISPR-based biosensors in pathogenic biosafety analysis.

Keywords: point-of-care; SARS-CoV-2 variants; drug-resistance; fungi; parasites; viable bacteria

1. Introduction

Pathogenic biosafety concerns have been growing in parallel to human progress and development (Jiang *et al.* 2021; Xiao *et al.* 2021). With economic globalization, countries over the world are interdependent on their supply and trade contacts, thus easily resulting in biosafety issue such as pathogen-caused. Pathogens, mainly including bacteria, viruses, fungi and parasites, are widely distributed in food and environment, and can cause both diseases and economic losses (Fraiture *et al.* 2017; Liu *et al.* 2021b; Shin *et al.* 2022; Yang *et al.* 2020; Yang *et al.* 2021b). For example, the Centers for Disease Control and Prevention (CDC) reported that human salmonellosis may cause approximately ~450 deaths out of ~1.2 million diseases per year in the United States, which often accompanied by clinical systemic symptoms with 8-72 h, such as bacteremia, fever and severe diarrhea (Farka *et al.* 2016; Yang *et al.* 2022b). The World Health Organisation (WHO) statistics showed that the current SARS-CoV-2 outbreak around the world has infected nearly 649 million people, of which over 6.65 million have died, as of December 20, 2022 (WHO 2022a). In 2020, there were an estimated 241 million confirmed cases and 627 thousand deaths from malaria globally (WHO 2022b). Pathogenic propagation inhibition and elimination require reliable and widely available detection tools. Most of the current methods to detect pathogens rely on culture and colony counting, quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA) (Xiong *et al.* 2020a). However, their high-cost or long turnaround time severely limits their applications, particularly in resource-constrained settings (Harshit *et al.* 2017; Li *et al.* 2019a; Randazzo *et al.* 2018; Xiong *et al.* 2020a).

The key to improve speed, accuracy and specificity of analytical technologies for pathogenic biosafety lies on developing new recognition and signal transduction patterns. A promising technology is the use of clustered regularly interspaced short palindromic repeat (CRISPR)-associated (CRISPR/Cas) systems. Because of its remarkable target sequence specificity, flexibility of design and ease of use, a myriad of biosensors based on CRISPR systems have been recently developed for POCT diagnosis, food and environmental safety analysis (Dai *et al.* 2020; Freije and Sabeti 2021; Li *et al.* 2019b; Li *et al.* 2022a; Li *et al.* 2022b; Yin *et al.* 2021b).

In this article, we first reviewed the mechanism and properties of class II CRISPR/Cas system, and discussed how CRISPR tools (Cas9, Cas12 and Cas13) have been leveraged for molecular assays for detecting nucleic acid and non-nucleic acid biomarkers (Fig. 1) (Chen *et al.* 2018; Gootenberg *et al.* 2018; Gootenberg *et al.* 2017; Harrington *et al.* 2018b; Ishino *et al.* 1987; Lee *et al.* 2020; Zhu *et al.* 2014). We then highlighted the strategies for CRISPR-based visual biosensing that capable of POCT diagnosis, such as the readouts using lateral-flow assay (LFA) papers and smartphones. We next summarized the CRISPR-based biosensing applications in pathogenic biosafety analysis including bacteria, viruses, fungi, parasites and their variants, and highlighted the analysis of pathogens' genotypes or phenotypes, such as viability and drug-resistance. Finally, challenges and opportunities of the CRISPR-based biotechnologies for pathogenic biosafety are also discussed.

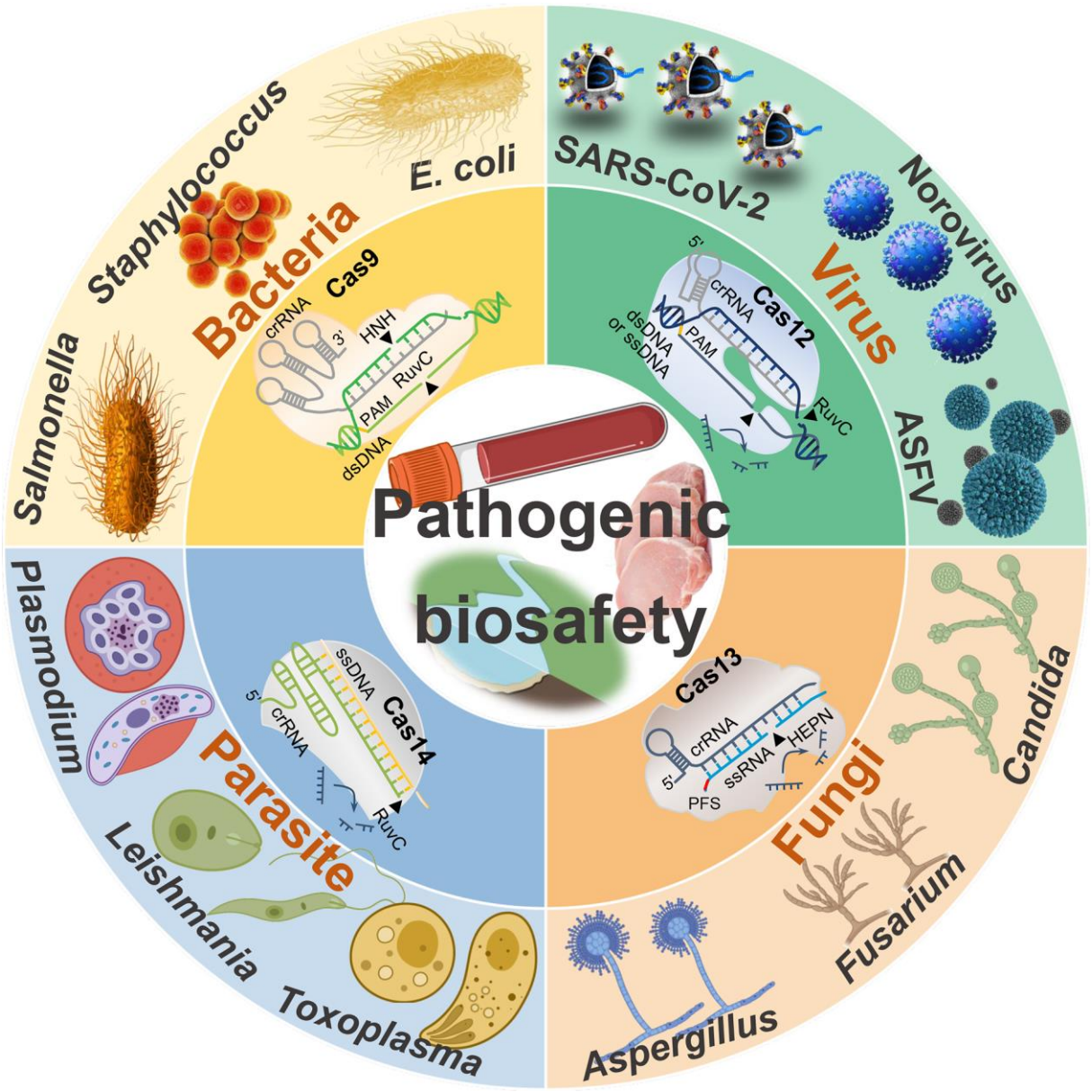


Fig. 1. Schematic diagram of advances of CRISPR-based biosensors for pathogenic biosafety. CRISPR/Cas systems include Cas9, Cas12, Cas13 and Cas14; pathogens include bacteria, viruses, fungi, parasites and their variants.

2. Principle of CRISPR-based biosensors

Following the discovery of unusual sequences of repeated DNA in *E. coli* (Ishino *et al.* 1987), researchers soon found their role in the adaptive immune system of about 87% of archaea and 47% of bacteria (Mojica *et al.* 2005). The type of sequences, known as

CRISPR, recognizes exogenous nucleic acids and subsequently cut with Cas endonucleases (Barrangou and Marraffini 2014; Jansen *et al.* 2002; Nouri *et al.* 2021; Stout *et al.* 2017). Two essential components, a CRISPR RNA (crRNA) and Cas protein, underlie the foundation of the CRISPR/Cas systems. The crRNA guides Cas endonuclease toward a specific DNA or RNA sequence of interest via complementary hybridization, triggering the cleavage activity on target nucleic acids (*cis*-) or non-target collateral ones (*trans*-) (Eş *et al.* 2019). The target regions in class II CRISPR systems, which widely used in applications of pathogen monitoring, are restricted to the vicinity of a protospacer adjacent motif (PAM) or protospacer flanking sequence (PFS). Cas effectors in class II CRISPR systems are widely used for molecular analysis and can be divided into two categories: DNA- and RNA-targeting enzymes. The DNA-targeting enzymes encompass Cas9 and Cas12 (Harrington *et al.* 2018a; Li *et al.* 2019d; Yin *et al.* 2020), both of which are limited to PAM motifs, while the RNA-targeting enzyme Cas13 needs PFS sequences (Gootenberg *et al.* 2017).

Activation of the target-dependent multiple-turnover *trans*-cleavage of Cas effectors via hybridization to a complementary target sequence enables CRISPR tools for a specific signal amplification of sequence recognition. Therefore, CRISPR/Cas system presents immense potential to be used for constructing biosensors due to its capacity for target recognition and signal amplification.

2.1. Nucleic acid biomarkers

Pathogenic contaminants can be identified through nucleic acid detection against their genomic DNA or RNA (Fig. 2A). In general, coupling CRISPR/Cas systems with

nucleic acid amplification strategies, like nucleic acid sequence-dependent amplification (NASBA) (Pardee *et al.* 2016), polymerase chain reaction (PCR) (Wang *et al.* 2021a), recombinase polymerase amplification (RPA) (Gootenberg *et al.* 2017), rolling circle amplification (RCA) (Yang *et al.* 2021a) and loop-mediated isothermal amplification (LAMP) (Broughton *et al.* 2020), can dramatically enhance the sensitivity and specificity of nucleic acid detection.

Cas9, including its variants, was the first CRISPR tool for constructing nucleic acid assays, targeting double-stranded DNA (dsDNA) (Bao *et al.* 2020; Pardee *et al.* 2016; Zhang *et al.* 2017; Zhou *et al.* 2018). NASBA-CRISPR cleavage (NASBACC), a Cas9-based strategy, combined toehold switches for the readout (Fig. 2B) (Pardee *et al.* 2016). A toehold trigger and Cas9-based specific cleavage were introduced into NASBA amplification. Cas9-mediated cleavage could be activated in the presence of a PAM sequence in the dsDNA produced from reverse transcription (RT), leading to a fragmented RNA without the toehold trigger. On the contrary, the NASBA-amplified full-length RNA contained the trigger, in the absence of the PAM, activated the toehold switch, as indicated by a colorimetric readout. The biosensing strategy yielded a high sensitivity (femtomolar range) and was capable of differentiating the arboviruses Dengue (DENV) from the Zika virus (ZIKV). The nCas9, with a single amino acid mutation in either the HNH (Cas9H840A) or RuvC (Cas9D10A) domain, can also play a nickase role; in the first case, it retains only the RuvC activity and therefore it only nicks target strand, and in the other case, only the non-target strand would be nicked (Gasiunas *et al.* 2012; Ran *et al.* 2013; Zhou *et al.* 2018). In 2018, a nCas9-based method

named CRISDA (CRISPR-nCas9-triggered nicking endonuclease-mediated strand displacement amplification) that enabled exponential strand displacement amplification (E-SDA) without heating steps was reported (Fig. 2D). 3’ overhang contained primers bind to the nicked strand, E-SDA was then started with the aid of a DNA polymerase and ssDNA binding proteins (SSBs). CRISDA exhibited high sensitivity in the attomolar range and specificity of single-nucleotide discrimination (Zhou *et al.* 2018).

A well-known tool, which utilized for Cas12 enzymes for nucleic acid detection was named DNA endonuclease-targeted CRISPR *trans* reporter (DETECTR) (Chen *et al.* 2018) (Fig. 2C, bottom). Other DETECTR-derived techniques included Cas14a-DETECTR, which required complementarity in the seed region for ssDNA substrate recognition, and exhibited higher fidelity than Cas12a-DETECTR for DNA single-nucleotide polymorphism (SNP) discrimination (Harrington *et al.* 2018a). SARS-CoV-2 DETECTR achieved a short sample-to-result time of ~45 min that was markedly shorter than that using RT-qPCR (4 h), and presented a high sensitivity (10 copies per μL) when combined with LAMP preamplification (Broughton *et al.* 2020).

The RNA-targeting enzyme Cas13 has been combined with nucleic acid preamplification to form biosensing platforms called specific high-sensitivity enzymatic reporter unlocking (SHERLOCK), which allowed rapid and sensitive sensing of desired DNA or RNA sequences (Fig. 2C, top) (Gootenberg *et al.* 2017; Kellner *et al.* 2019). SHERLOCK could be used to determine ZIKV ssRNA with a limit of detection (LOD) of ~2 aM, which is 7 times more sensitive than Cas13a alone and enables SNP detection (Gootenberg *et al.* 2017). In addition, its sibling method called SHERLOCKv2 could

achieve a multiplexing test. Four Cas effectors, AsCas12a, LwaCas13a, CcaCas13b and PsmCas13b, were used to construct four independent signal channels for simultaneous multi-target detection (Gootenberg *et al.* 2018).

Nevertheless, target preamplification makes CRISPR-based biosensors time-consuming and susceptible to be interfered with sample matrices. The rational design of cascade CRISPR assays can enable preamplification-free sensing (Fig. 2E). For example, a cascade CRISPR/Cas assay (casCRISPR) which coupled Cas13a with Cas14a has been developed, allowing amplification-free, fM-sensitive and SNP-specific detection of RNA. Different versions of casCRISPR have been created. When comparing Cas13a-Cas12a (casCRISPR v2) with Cas13a-Cas14a (casCRISPR v1), it was shown that casCRISPR v1 performed better on the LOD of ~6.25 fM while it was ~100 fM for casCRISPR v2 (Sha *et al.* 2021). Furthermore, a hybrid CRISPR/Cas that fused Cas12a and Cas13a to a single protein enabled to simultaneously target DNA and RNA in a single tube. The hybrid CRISPR/Cas eliminated the use of multi-enzymes, which can address the drawbacks in the incompatible reaction conditions (Guk *et al.* 2023). CRISPR/Cas-only amplification network (CONAN) requiring no nucleic acid amplification can be achieved for the isothermally sensitive detection of genomic DNA (Shi *et al.* 2021). Rationally integrating Cas12a-crRNA with reporter into a switchable sgRNA achieved a positive feedback circuit-caused exponential dynamic, enabling an aM-sensitivity against the genomic DNA of hepatitis B virus. In addition, an auxiliary CRISPR-associated enzyme, termed Csm6, has attracted interest. Combining Csm6 with

Cas13a in a cascade increased the signal sensitivity for nucleic detection by ~3.5 times compared to that only using Cas13a (Gootenberg *et al.* 2018; Liu *et al.* 2021a).

The features of simplicity, programmability, compatibility, specificity and the capability in signal amplification make CRISPR/Cas tools outperform other natural or engineered molecular scissors, such as restriction endonucleases or deoxyribozymes (DNAzymes). Despite the advantages in dealing with nucleic acid targets, a predominant challenge for CRISPR-based assays is the fact that non-nucleic acid biomarkers are also critical in pathogenic biosafety. Confronting this challenge requires the development of signal transduction mechanisms that convert non-nucleic acid recognition into nucleic acid switch.

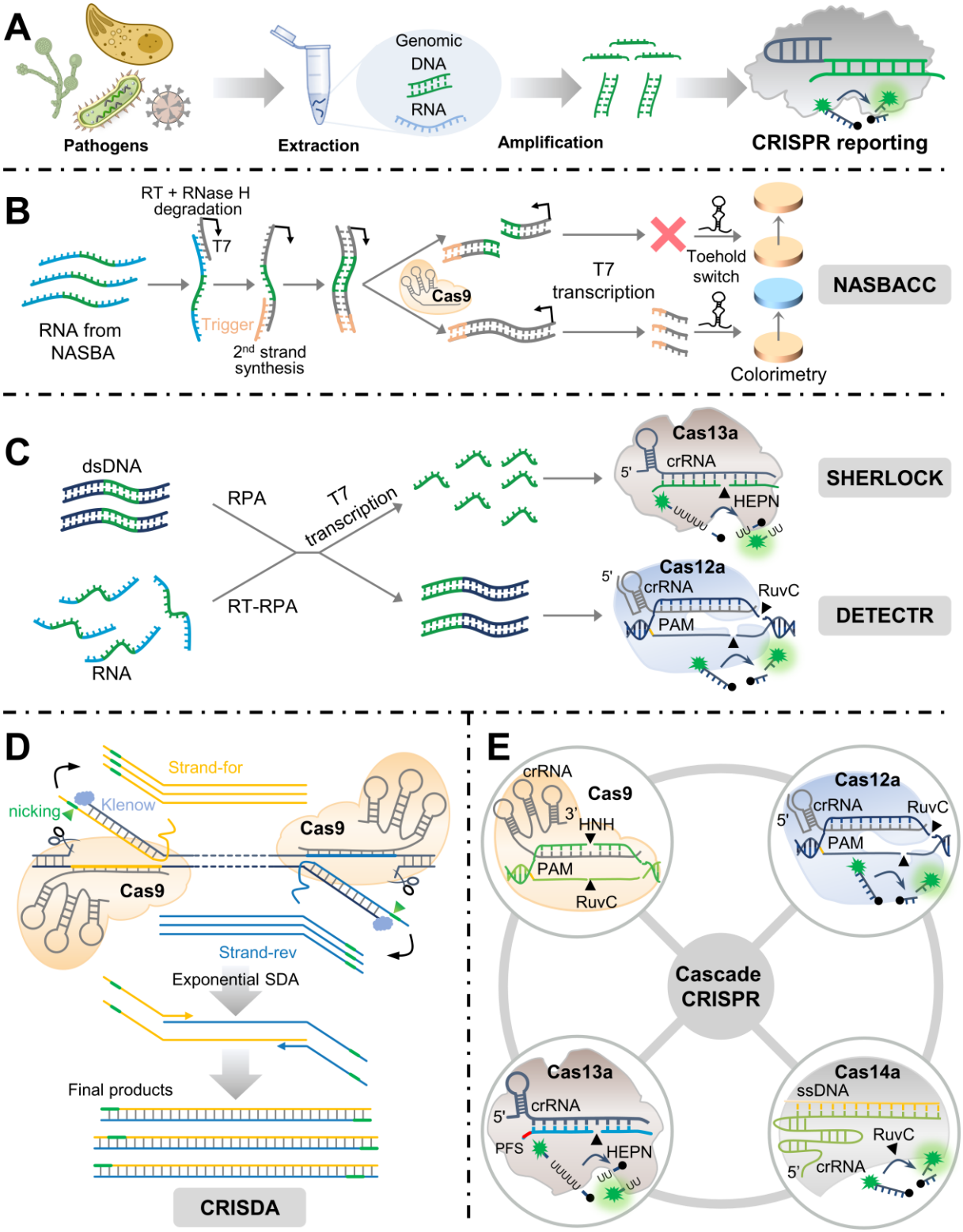


Fig. 2. CRISPR/Cas-based biosensors for pathogenic nucleic acid biomarkers. (**A**) Nucleic acid targets (genomic DNA or RNA) from pathogens need to be extracted, and being detected by CRISPR reporting. (**B**) NASBACC assay (Pardee *et al.* 2016). RNA targets from NASBA amplification

started with RT, then RNase H degraded the RNA from the RT-derived RNA/DNA hybrid. The binding of the complementary DNA to a primer that appended a trigger element for the toehold color sensor activates the 2nd DNA strand synthesis. Cas9-based cleavage truncated the dsDNA template that contained a PAM motif for T7 transcription, leading to no activation of toehold sensor, while producing a visible change without the PAM. (**C**) SHERLOCK and DETECTR assay (Chen *et al.* 2018; Gootenberg *et al.* 2017). RNA-targeting Cas13a, the amplified products needed to be T7-transcribed into RNA. The amplicons (DNA or RNA) bound to the guided-RNA to activate the Cas12a (used in DETECTR) or Cas13a (used in SHERLOCK) and subsequently triggered the fluorescent reporting. (**D**) CRISDA assay (Zhou *et al.* 2018). A pair of Cas9(H840A) enzymes as nickase were employed to recognize and nick each border of target DNA. A pair of nicking site-contained primers bound to the exposed non-target strand and trigger the exponential SDA amplification with the assistance of SSBs, generating a mass of expected final products (Strand-for and Strand-rev). (**E**) Cascade CRISPR assay (Sha *et al.* 2021; Shi *et al.* 2021). Combination of Cas9, Cas12a, Cas13a and Cas14a in cascade applications was expected to increase sensitivity without the need for preamplification.

2.2. Non-nucleic acid biomarkers

Multitudes of analytes related to pathogenic biosafety are non-nucleic acid targets, such as the entire pathogenic cells, specific antigens and antibodies, which cannot be directly detected by CRISPR-driven assays. However, tools for molecular recognition or signal transduction, such as antibodies, functional nucleic acids (aptamers or DNAzymes) and other molecular translators (Li *et al.* 2021a; Li *et al.* 2021c; Lin *et al.* 2019; Peng *et al.* 2018), enable CRISPR/Cas system to be used for detection of non-

196 nucleic acid biomarkers (Fig. 3A). Translators for metal ions or chemical analytes can
197 be artificially selected via an *in vitro* process (systematic evolution of ligands by
198 exponential enrichment, SELEX) (Deng *et al.* 2014; Peng *et al.* 2018).

199 CRISPR/Cas system can be combined with traditional ELISA platforms (Fig. 3B),
200 which may avoid complicated chemical modifications and laborious purification steps
201 for traditional ELISA (Acharya *et al.* 2013; Bruch *et al.* 2019). Li group described an
202 immunoassay based on aptamer-linked CRISPR/Cas12a for detecting non-nucleic acid
203 analytes (Li *et al.* 2021c). Antigen-aptamer complex as the linker was divided into two
204 parts, one of which for the capture module construction and another one for the aptamer-
205 linked CRISPR/Cas12a module. The platform yielded a higher sensitivity than ELISA
206 alone for protein (carcino-embryonic antigen), bacteria (*Escherichia coli* O157:H7, *E.*
207 *coli* O157:H7) and virus (norovirus) analysis.

208 Additionally, DNAzyme, a single-stranded DNA with high catalytic activity,
209 promises for bacteria detection when integrated with the CRISPR/Cas systems (Fig. 3C).
210 CIM (crude intracellular mixture) and CEM (crude extracellular mixture) could be used
211 for direct detection of bacteria (Ma *et al.* 2021c). DNAzymes were *in vitro* selected for
212 the recognition of *Clostridium difficile* (Shen *et al.* 2016), *E. coli* (Ali *et al.* 2011) and
213 *Helicobacter pylori* (Ali *et al.* 2019), have been selected *in vitro*. In the DNAzyme assay,
214 the 3'-termini of substrate labeled with a biotin that can bind to the fixed streptavidin,
215 and the substrate contained a DNA activator sequence for Cas12a at 5' termini. The
216 substrate-enzyme duplex remains stable due to its higher melting temperature, while a
217 low melting temperature between the DNA activator and the DNAzyme, ensured the

detachment of the DNA activator at room temperature (Xiong *et al.* 2020b). In the presence of target CEM or CIM of bacteria, DNAzyme cleaved a substrate and the DNA activator sequence was released, then Cas12a became activated and cut the fluorescent DNA reporters.

Aptamers, single-strand oligonucleotides, are able to be used as conformational switching motifs to construct ligand-responsive strategies in combination with CRISPR/Cas. Xie and colleagues reported a CRISPR/Cas12a-based bacteria detection platform based on a magnetic separation process (Li *et al.* 2020). The aptamer (Ab-Apt) of *Acinetobacter baumannii* (Ab) was immobilized on the surface of magnetic beads (MBs) via biotin-streptavidin interaction. A Cas12a activator-contained strand (c-Ab-Apt) was introduced to partially hybridize to the aptamer. The c-Ab-Apt would be released from the MBs in the presence of Abs, thus triggering the activation of Cas12a to output fluorescent signal. The LOD was estimated to be ~3 CFU/mL, which was close to the single-cell level. The magnetic separation, however, makes the experimental procedure complicated. The integration of two-dimensional materials with CRISPR/Cas systems could eliminate the magnetic separation process. Zhang *et al.* developed a rapid assay through coupling MXene with Cas12a for bacteria detection (Fig. 3D) (Sheng *et al.* 2021). The ASI/CSI probe consists of the target aptamer strand I (ASI) and corresponding complementary strand I (CSI), in which ASI contained the activating sequence for Cas12a to cut the ssDNA reporter. This turn-off strategy with ultralow background can quantify bacteria with detection limit of 23 CFU/mL.

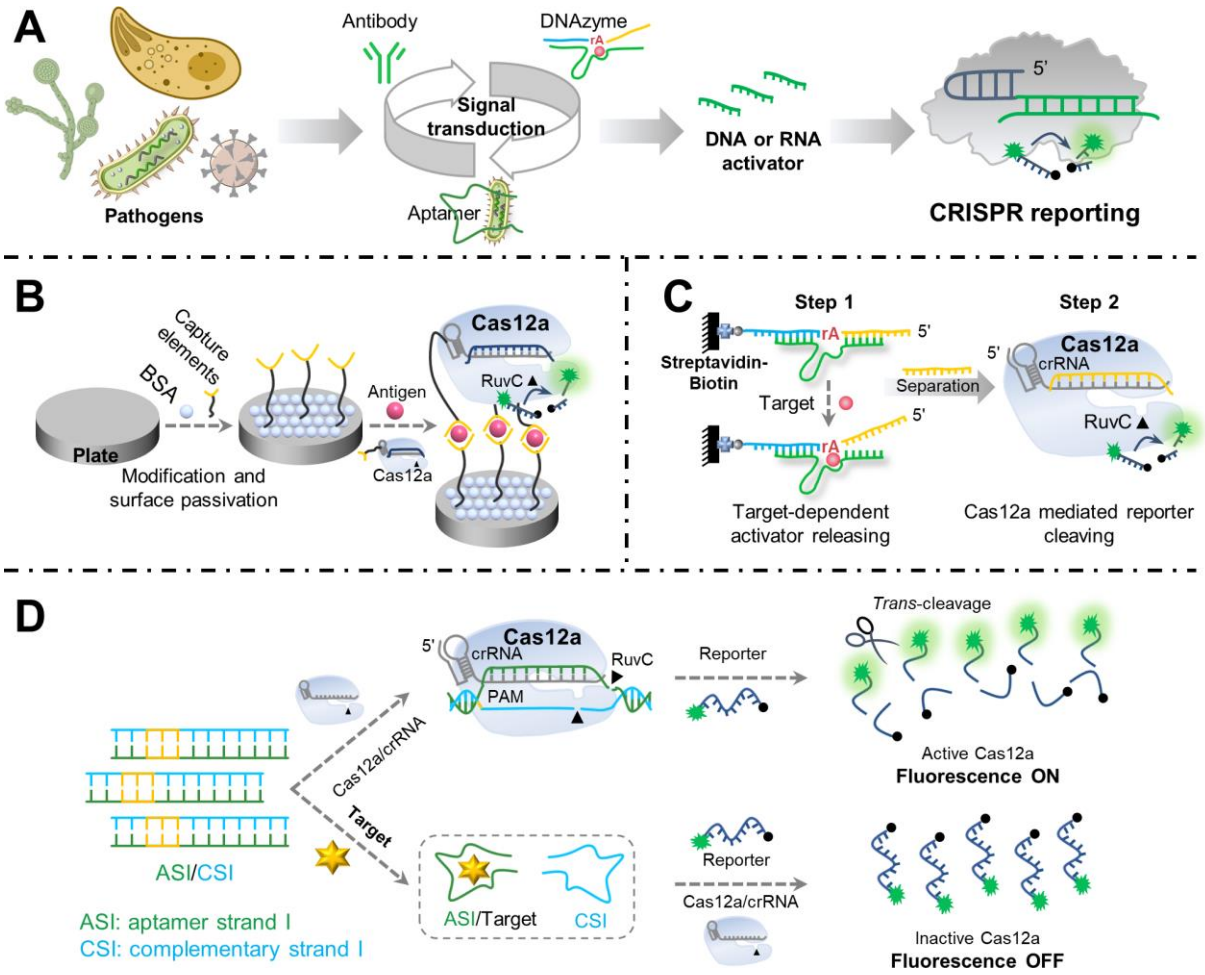


Fig. 3. CRISPR/Cas-based biosensors for analysis of pathogenic non-nucleic acid biomarkers. (**A**) Non-nucleic acid biomarker from pathogens needed to be recognised and converted into nucleic acid switch signals that can activate the subsequent CRISPR reporting process. (**B**) Antibody as the signal transduction element (Li *et al.* 2021c). Aptamer-linked Cas12a-based immunoassay for detection of non-nucleic acid analytes. (**C**) DNAzyme as the signal transduction element (Xiong *et al.* 2020b). DNAzyme regulates Cas12a sensors for pathogen detection. (**D**) Aptamer as the signal transduction element (Li *et al.* 2020). Combination of aptamer and Cas12a based versatile platform for sensitive bacteria quantification.

3. Strategies for POCT diagnosis

The direct capture of nucleic acids using Cas endonucleases without complicated purification and denaturation steps makes CRISPR tools compatible with POCT diagnosis, as they can rapidly provide easy-to-interpret results without sophisticated equipment. The fast and cost-effective readouts including fluorometry, colorimetry and lateral flow immunochromatography, were preferred for POCT diagnosis, achieving visual measurement with the assistance of LFA papers and mobile phones (Fig. 4) (Fozouni *et al.* 2021; Zhang *et al.* 2022a).

CRISPR-based fluorescent assays output signals via the collateral cleavage of ssDNA or ssRNA reporters carrying a pair of adjacent fluorophore and quencher, and the resulting fluorescence can be visualized by the naked eye under blue light (Fig. 4A, top). Inspired by that, Wang *et al.* proposed a Cas12a-based one-pot isothermal detection method (named Cas12aVDet) for on-site analysis of nucleic acids (Wang *et al.* 2019). Cas12VDet can accomplish the entire detection process within 30 min, and circumvent the uncapping contamination. Mobile phones are widespread, cost-effective, field-portable and easy to operate, thus are well suited for POCT diagnosis (Fig. 4A, bottom) (Kong *et al.* 2017; Vashist *et al.* 2014). Recently, an amplification-free Cas13a-based platform for on-site SARS-CoV-2 RNA detection without additional manipulation was developed and its signal could be read by a smartphone microscope (Fozouni *et al.* 2021). This assay, different from the other CRISPR-based techniques, did not require nucleic acid preamplification and multiple CRISPR/Cas cascade for signal amplification. Employment of multiple crRNAs targeting various loci can enhance Cas13a activity,

leading to an amplified signal output via multiple Cas13a proteins and their trans-cleavage activity. Combining with two crRNAs to target two loci markedly increased the detection sensitivity. Finally, it was proven that a mobile phone camera allowed 100 % accurate detection for 200 copies/μL and 50 % accuracy for 50 copies/μL over 30 min of measurement.

Colorimetric detection enables direct observation of the assay results with the naked eye. Metallic nanoparticles, such as gold nanoparticle (AuNPs) and silver nanoparticle (AgNPs), display distance- and size-dependent properties, thus are widely used in colorimetric biosensors (Mirkin *et al.* 1996). The CRISPR-mediated *trans*-cleavage of single-strand DNA/RNA linked to gold nanoparticles resulted in a vibrant colour in solution (Fig. 4B, top) (Zhou *et al.* 2023). Taking advantage of AuNPs to develop a programmable Cas12a-driven colorimetric coding strategy for highly rapid analysis of telomerase activity via naked-eye observation (Cheng *et al.* 2021). This assay involved three colorimetric codes that respond to positive, negative and false-negative results, respectively. Each code had two channels. Channel 1 contained Cas12a/crRNA1 in charge of distinguishing telomeric repeat DNA amplicons that indicated positive telomerase activity, and channel 2 contained Cas12a/crRNA2 responsible for monitoring internal control. Only lighting up of both two channels represented a positive result. Another strategy of colorimetric biosensors employs the enzymes (e.g., horseradish peroxidase, HRP) capable of catalyzing a chromogenic substrate (e.g., 3,3’,5,5’-tetramethylbenzidine, TMB). Use of HRP-labeled oligonucleotides immobilized on the surface of magnetic beads serving as the reporter of Cas effectors can achieve target-

triggered colorimetric signal output (Fig. 4B, bottom) (Samanta *et al.* 2022). Benefitting from the high catalytical efficiency of HRP, the HRP-reporting CRISPR assay exhibited quick responsiveness (<1 h) and ~30-fold higher sensitivity (~10 fM) than that using conventional fluorophore/quencher reporter.

Colorimetric visualization can also be achieved by a chromogenic reaction. CRISPR processing produces a primer to initiate the subsequent amplification, leading to abundant G-quadruplex/hemin complexes (G4/hemin) that can convert the colorless ABTS-H_2O_2 to green color (Fig. 4C). A PAMmer-assisted Cas9 system specifically recognized and cleaved the target single-stranded nucleic acid, releasing a product fragment that can serve as the primer to activate subsequent exponential amplification reaction (EXPAR). G-rich EXPAR products were assembled with hemin to achieve a colorimetric detection (Wang *et al.* 2021b; Yin *et al.* 2021a). Moreover, Spoelstra *et al.* reported a CRISPR-assisted visual assay based on turbidity for nucleic acid detection, allowing to directly read the results by naked eyes in a tube (Spoelstra *et al.* 2021). In this assay, long oligonucleotides at high concentration caused the solution turbid and the turbidity can significantly decrease when the long oligonucleotides were largely cleaved by activated Cas proteins.

LFAs allow simple visualization using a reporter carrying fluorescein and biotin bound to anti-fluorescein isothiocyanate (FITC) antibodies coupled to gold nanoparticles in the sample pad of the strip (Fig. 4D). By coupling with LFAs, the Cas13a-based SHERLOCK platform allowed visual detection of ZIKV and DENV in patient samples

at a low concentration of 1 copy per microliter, and it enabled to detect Dengue virus from patient samples with no need for instrument in 2 h (Myhrvold *et al.* 2018).

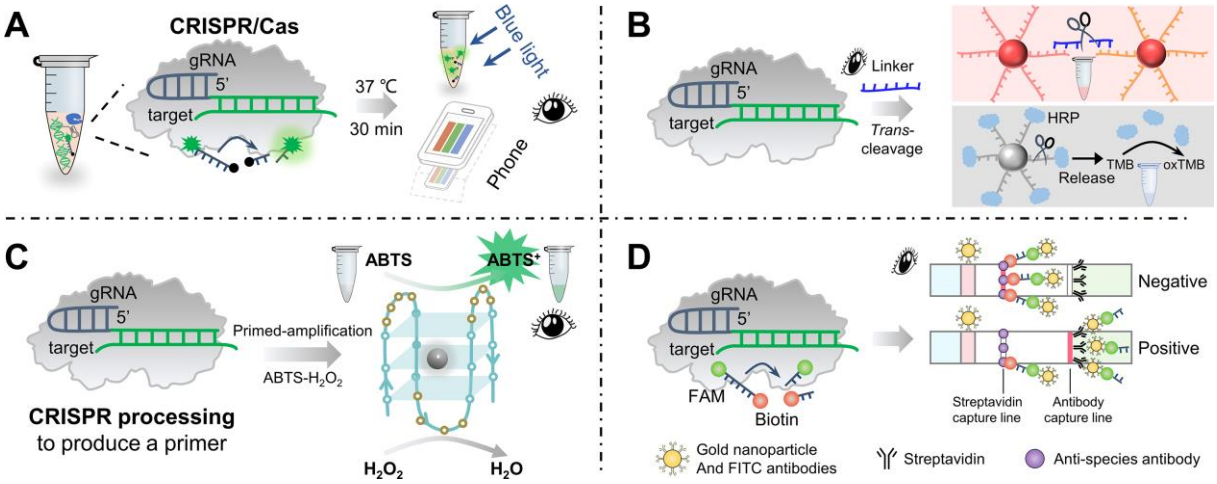


Fig. 4. Strategies for CRISPR-based POCT diagnosis. (**A**) CRISPR-based visual assay via fluorescence under a light excitation, the reporting signal can be observed by naked eyes or smartphones (Fozouni *et al.* 2021; Wang *et al.* 2019). (**B**) The CRISPR-mediated *tans*-cleavage towards the single-strand DNA/RNA linkers which behave as the junctions between gold nanoparticles or between magnetic beads and HRP, resulting in a colour change in solution (Cheng *et al.* 2021; Samanta *et al.* 2022). (**C**) CRISPR processing produces a primer to initiate the subsequent amplification, leading to produce abundant G4/hemin complexes that can catalyze the conversion of colorless ABTS-H_2O_2 into green (Wang *et al.* 2021b; Yin *et al.* 2021a). (**D**) LFAs contribute a simple visualization via taking advantage of reporters carrying fluorescein and biotin bind to anti-fluorescein isothiocyanate (FITC) antibodies coupled to gold nanoparticles in the sample pad of the strip (Myhrvold *et al.* 2018).

4. Pathogenic biosafety

A number of pathogens have been widely identified in clinical, food and environmental samples, serve as a critical biosafety issue that severely threatens human

health (Al Mughairy and Al-Lawati 2020). In order to effectively control their presence in clinic, food and environment, accurate and rapid analytical methods for monitoring these pathogens are in need of being developed and implemented. CRISPR-based analytical techniques have been widely exploited for pathogen diagnosis (Xia *et al.* 2021; Xiao *et al.* 2021). In this section, we highlighted current CRISPR-based biosensors for detecting bacteria, viruses, fungi and parasites, and their contributions to control pathogenic biosafety.

4.1. Bacteria

Information of pathogen's phenotypes and genotypes, such as the viability and drug-resistance, is important for estimating biosafety risks. In this section, we reviewed current CRIPSR-based biosensors for detecting bacteria as well as their key phenotypes and genotypes via detecting nucleic acid biomarkers with single-nucleotide resolution.

4.1.1. Bacteria cell

Predominantly, genetic testing has been considered the most reliable way for bacteria detection. Coupling with nucleic acid amplification, CRISPR-based assays allow a highly sensitive detection of bacteria and their genetic markers. Ascribing to the compatible condition of RPA amplification and Cas12a cleavage, the Cas12a-based RPA assay has been applied for the detection of bacteria with different sensing strategies, such as fluorescence (Wang *et al.* 2023), surface-enhanced Raman scattering (SERS) (Zhuang *et al.* 2022) and LFA (Liu *et al.* 2022a). For example, LFA-coupled assays for on-site detection of *Salmonella typhimurium* (*S. typhimurium*) (Zhuang *et al.* 2022) and

Staphylococcus aureus (*S. aureus*) (Liu *et al.* 2022a), and an one-pot fluorescent assay for POC testing of *Enterocytozoon hepatopenaei* infection in shrimp (Wang *et al.* 2023). The ratiometric biosensing can alleviate the cause of false-positive or negative results by measuring dual signals. Ma *et al.* adopted silver nanoclusters (AgNCs) as outputs to construct a Cas12a-based ratiometric fluorescent assay for detection of *S. typhimurium* (Fig. 5A) (Ma *et al.* 2023). A converter ssDNA was designed to complement the AgNCs probe, in which capable of converting green fluorescence emissive AgNCs to red emission. Degradation of converter ssDNA terminate the green-to-red fluorescent conversion, enabling ratiometric detection. This assay can detect *S. typhimurium* as low as 1 CFU/mL. Replacing Cas13a with Cas12a can evade the redundant transcription because of the DNA-targeted activation of Cas12a. Wei *et al.* coupled Cas12a with Ag^{+}-3,3',5,5'-tetramethylbenzidine (TMB) chromogen to construct a convenient colorimetric reaction for visual detection of *S. aureus* (Fig. 5B) (Wei *et al.* 2022a), posing the potential for POC application. The Ag^{+}-TMB which was pre-coupled to polycytosine-loaded magnetic beads served as the substrate of *trans*-cleavage by Cas12a.

Besides, using aptamers to directly recognize target bacteria followed by converting to nucleic acid signals allows to detect whole bacteria cells. An APC-Cas (allosteric probe-initiated catalysis and CRISPR-Cas13a) assay was established by employing Cas13a combined with a processed aptamer to detect *Salmonella enteritidis* cells in drinking water and milk (Shen *et al.* 2020). The allosteric probe contained three domains: aptamer domain, primer recognition domain and T7 promoter domain. In the presence of bacteria cells, the allosteric probe would unfold to unveil the primer binding site

domain and yielded dsDNA under the participation of DNA polymerase, followed by the release of target bacteria to bind the succeeding allosteric probe. Then, transcription amplification was conducted through the match between T7 promoter domain and T7 RNA polymerase, generating numerous ssRNAs which could activate CRISPR/Cas13a to cleave ssRNA reporters. The triple amplification of multiple-turnover recognition, transcription and Cas13a cleavage endows the APC-Cas system with single-molecular sensitivity.

4.1.2. Viable bacteria

Conventional culturing methods for viable pathogenic bacteria identification may fail due to the unculturability of viable but non-culturable (VBNC) pathogens. In addition, DNA targeting techniques may also fail to indicate whether the pathogen is alive or not because of the long-term existence of genomic DNA (Sheng *et al.* 2019; Trieu and Lee 2019; Váradi *et al.* 2017). RNA in dead cells is rapidly degraded, thus the presence of cellular RNA can indicate live bacteria. The emergence of RNA-targeting Cas13a opens possibilities for sensing applications in viable bacteria. Zhang *et al.* recently reported a light-up RNA aptamer signaling-Cas13a-based assay for label-free detection of viable pathogenic bacteria (*Bacillus cereus*) by directly targeting pathogen RNAs, achieving nucleic acid amplification-free and one-pot detection within 20 min (Fig. 5C) (Zhang *et al.* 2021). The introduction of a light-up RNA aptamer, broccoli contributed to eliminate the need for chemically labeled RNA reporters, and allowed reverse transcription-free analysis of RNA targets. Furthermore, combining NASBA preamplification with Cas13a

detection allowed to improve the sensitivity down to 1 CFU and 1 % viable bacteria (Xue *et al.* 2022).

Acquirement of spatial information of microbes and intra/inter-cell is conducive to understand the organization of microbial communities and cellular behaviors and functions such as antimicrobial resistance. *In vivo* imaging of bacterial genotype can provide insight into the complex microbiota and pathogenic risk (Shi *et al.* 2020). A polyethyleneimine-encapsulated CRISPR/Cas12a-circular reporter nanoprobes (CasCLR) tool was designed for rapid and direct *in vivo* visualization of genomic information in living bacteria (a clinically isolated *E. coli*) (Fig. 5D) (Li *et al.* 2022c). To enhance the stability of nucleic acid reporter in living bacteria, a circularization strategy was adopted, and circular reporter could be cleaved by Cas12a. Circularization of reporter improved signal-to-noise ratio by 14.5-fold than that in linearity. CasCLR enabled direct analysis of pathogenic bacteria in uncultured gut microbe of mouse.

The specific binding to the active proteins on the surface of viable bacteria cell enables aptamer to indicate the living bacteria state. An aptamer-based Cas14a platform for amplification-free detection of viable *S. aureus* with no need for nucleic acid extraction was developed (Fig. 5E) (Wei *et al.* 2022b). The binding of specific aptamer to viable *S. aureus* would release the blocker DNA from the pre-hybridized aptamer-blocker complex. The released blocker DNA could trigger the *trans*-cleavage of Cas14a under the guidance of sgRNA, generating a positive fluorescent signal. It allowed a low LOD of 400 CFU/mL viable bacteria cells and 100% detection accuracy in complex matrix comparable to qPCR.

4.1.3. Drug-resistant bacteria

The widespread prevalence of antimicrobial-drug-resistant bacteria resulted from the overuse of antibiotics. A magnetic relaxation switching strategy (C-MRS) based on Cas12a was demonstrated for the detection of methicillin-resistant *S. aureus* (MRSA) in food samples (Wei *et al.* 2023). Benefitting from the high catalytic activity of alkaline phosphatase, the C-MRS assay achieved preamplification-free detection. And the sequence specificity of Cas12a endowed C-MRS with the capacity of discriminating single-nucleotide variations. Capturing the cell information of spatial pattern and heterogeneity is the key approach to investigate cellular behaviors and functions such as drug-resistance. Xia *et al.* utilized the collateral cleavage of Cas12a to achieve single-cell *in situ* analysis of drug-resistant *Salmonella* by targeting the non-repetitive gene, *oqxB* (Xia *et al.* 2023). It was found that simultaneously targeting multiple loci could enhance the positive imaging signal, contributing to robust imaging of drug-resistant strains isolated in the clinic and intestinal tract sections. In addition, the *in situ* analysis assay allowed for investigation of the resistance of *Salmonella* towards to salt stress, and revealing a higher survival advantage of drug-sensitive strains than that drug-resistance strains.

Besides the plasmid-mediated drug-resistant bacteria, detection of SNP-involved drug-resistant strain could be more challenge. Yang *et al.* constructed a Cas12a-signaling amplification refractory mutation system (ARMS)-PCR (termed cARMS) assay, achieving specific and sensitive detection of SNP-involved *Salmonella enterica* (*S. enterica*) (Fig. 5F) (Yang *et al.* 2022b). High specificity of SNP recognition relied on

ARMS module which used an allele-specific forward primer with an artificial mismatch at the 4st position of the 3'-end. The following PCR amplification and Cas12a signaling conferred the cARMS assay with a sensitivity down to ~0.5 % comparable to qPCR. Particularly, the cARMS assay was applied to investigate the resistance of the drug-resistant bacteria to salt stress, and found a higher adaptation of drug-resistant strain than drug-sensitive one.

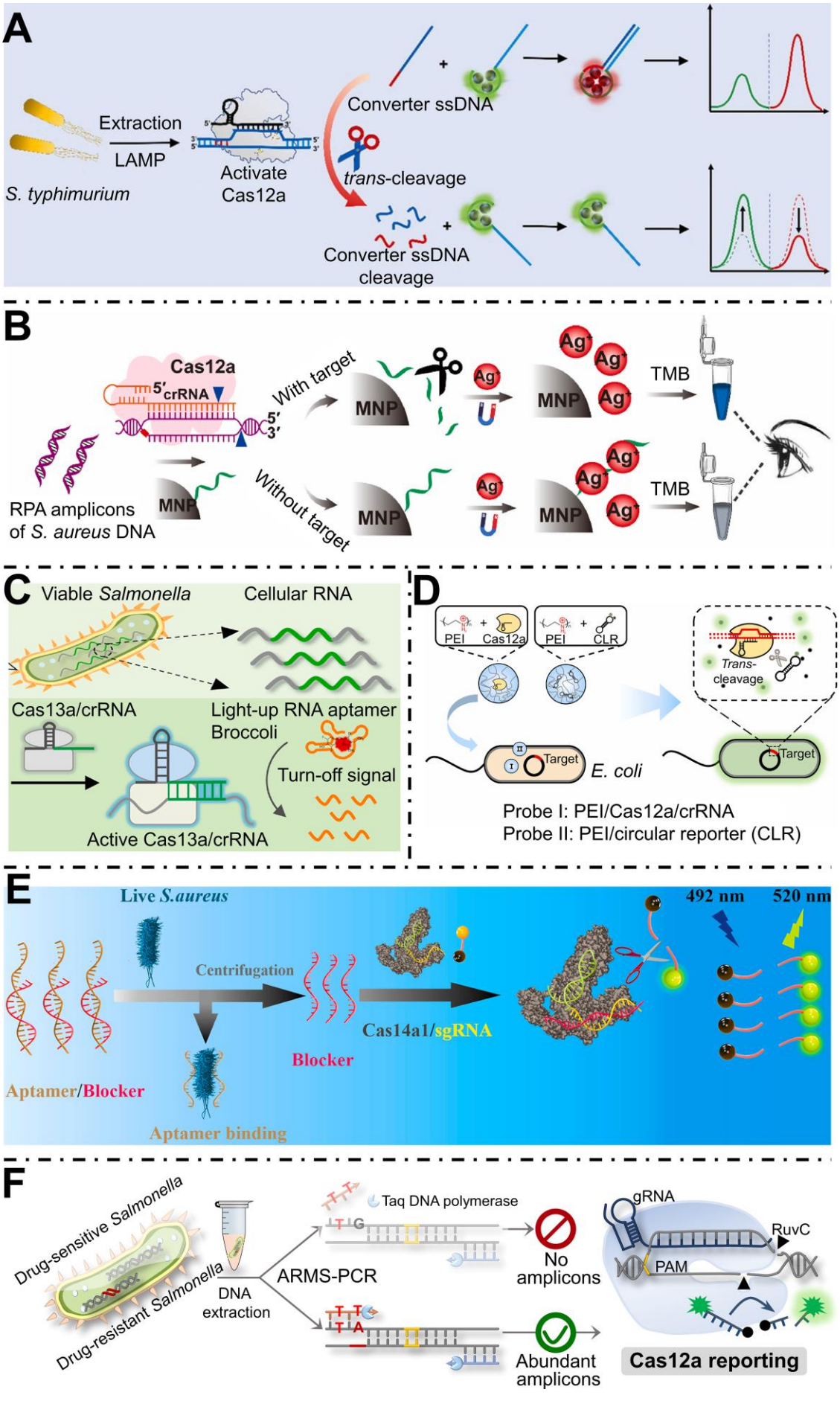

A
Extraction
LAMP
S. typhimurium
Activate Cas12a
Converter ssDNA
trans-cleavage
Converter ssDNA cleavage
B
Cas12a
5′ crRNA
5′ 3′
With target
Without target
MNP
Ag+
TMB
RPA amplicons of S. aureus DNA
C
Viable Salmonella
Cellular RNA
Cas13a/crRNA
Light-up RNA aptamer Broccoli
Turn-off signal
Active Cas13a/crRNA
D
PEI
Cas12a
CLR
Trans-cleavage
Target
E. coli
Probe I: PEI/Cas12a/crRNA
Probe II: PEI/circular reporter (CLR)
E
Live S.aureus
Centrifugation
Cas14a1/sgRNA
Blocker
Aptamer/Blocker
Aptamer binding
492 nm
520 nm
F
Drug-sensitive Salmonella
Drug-resistant Salmonella
DNA extraction
ARMS-PCR
Taq DNA polymerase
No amplicons
Abundant amplicons
gRNA
RuvC
PAM
Cas12a reporting

Fig. 5. CRISPR-based biosensors for detecting pathogenic bacteria. (A) AgCNs-based Cas12a assay for ratiometric detection of *S. typhimurium* (Ma *et al.* 2023). The dual green-to-red fluorescent outputs alleviated the cause of false-positive or negative results. (B) Colorimetric Cas12a assay using Ag^+-TMB reporter for detection of *S. aureus* (Wei *et al.* 2022a). The target-triggered color change of Ag^+-TMB reporter posed the potential for POC application. (C) Label-free Cas13a assay using light-up RNA aptamer broccoli as the reporter for direct detection of viable *Bacillus cereus* (Zhang *et al.* 2021). Use of RNA-targeting Cas13a evaded the application of RT. (D) Visualization of genomic information in living bacteria *E. coli* by Cas12a (Li *et al.* 2022c). Circularization of reporter improved the resistance to endonuclease in living cell. (E) An aptamer-based Cas14a platform for amplification-free detection of viable *S. aureus* (Wei *et al.* 2022b). Use of aptamer binding the active proteins on the surface of viable bacteria cell can indicate the living cellular state. (F) Cas12a signaling ARMS-PCR for the detection of SNP-involved drug-resistant *S. enterica* (Yang *et al.* 2022b). Dual recognition and amplification via ARMS-PCR and Cas12a binding enabled this assay high specificity.

4.2. Viruses

Pathogenic viruses can be grouped into two categories by the genetic material, DNA and RNA. DNA viruses include the grapevine red-blotch virus (GRBV) (Li *et al.* 2019c), white spot syndrome virus (WSSV) (Chaijarasphong *et al.* 2019) and African swine fever virus (ASFV) (He *et al.* 2020) that have been reported to be detected by CRISPR tools. Additionally, in the RNA viruses, norovirus is a foodborne virus that can causes over 90% of nonbacterial gastrointestinal diseases (Patel *et al.* 2009), particularly, the prevalent SARS-CoV-2 has been identified for person-to-person and material-to-person

transmission (Carducci *et al.* 2021; Yekta *et al.* 2021; Zhou and Shi 2021). Thus, developing techniques for detection of SARS-CoV-2 liked viruses is essential for identifying not only infected people but also contaminated foods and their surrounding environments.

4.2.1. DNA viruses

Considering the widespread infection of aquatic crustaceans with WSSV, Chaijarasphong *et al.* developed a Cas12a-based method for viral diagnosis by integrating nucleic acid amplification and Cas12a reporting. (Chaijarasphong *et al.* 2019). The described assay yielded a detection limit down to 200 copies WSSV per reaction and a sample-to-answer time of 1 h. Integrated visual reporting systems, such as color transition mediated by AuNPs, allows the Cas12a assays to be naked-eye detectable and field depolyable. The assay could achieve a detection limit of 40 pM, which was sufficient for the detection of GRBV infections in grapevine samples (Fig. 6A) (Li *et al.* 2019c). Nucleic acid preamplification may generate false-positive readouts resulted from nonspecific amplification and aerosol cross contamination, which severely impedes POCT application. Detection signal accumulation without nucleic acid preamplification can be achieved through the use of multiple crRNAs that target different loci. The high mortality (can reach 100 %) and outbreak rates of hemorrhagic African swine fever in both wild boars and domestic pigs make it urgent to exploit diagnostic technologies for ASFV control (Bai *et al.* 2019). Multiplex Cas12A/crRNA system enabled preamplification-free detection and showed ~64-fold improved

sensitivity (~1 pM) than the conventional single one for detecting ASFV (Fig. 6B) (Zeng *et al.* 2022). In combination with colorimetric module such as using urease catalysis, Cas12a detection can achieve a multiplexing and visual POCT diagnosis of ASFV with pM-sensitivity without nucleic acid preamplification (Fig. 6C) (Ki *et al.* 2022).

4.2.2. RNA viruses

Norovirus is a serious RNA virus that frequently occurs in public indoor spaces around the world mainly including schools and hospitals, through the consumption of Norovirus-contaminated food (Ahmed *et al.* 2014). Generally, RNA target first needs to be performed RT to obtain a corresponding DNA sequence. Qian *et al.* combined RT-RPA with Cas12a and LFA to develop a portable and reliable platform that enabled rapid (within 40 min) and sensitive (965 copies/mL) detection of human norovirus (Qian *et al.* 2021).

The global pandemic outbreak of 2019 novel coronavirus disease (COVID-19) makes an urgent demand for developing rapid, accessible and precise techniques for detection of the causative RNA virus, SARS-CoV-2 to reduce the risk of disease transmission. Dual-gene simultaneous detection is expected to improve accuracy and reduce false-negative results caused by partial gene digestion and amplification errors (Chen *et al.* 2022; Kim *et al.* 2020; Trémeaux *et al.* 2020). Such strategy has been developed for SARS-CoV-2 detection, where dual-Cas12a-based assay with the aid of an automated centrifugal microfluidics allowed on-site observation by the naked eye (Fig. 6D) (Chen *et al.* 2022). This "sample-to-answer" microfluidic platform can

automatically detect clinical SARS-CoV-2 samples with 100 % accuracy comparable to RT-PCR.

CRISPR-based detection technologies for viral variants of concern (VOCs), particularly the SARS-CoV-2 VOCs, have been sufficiently developed during the COVID-19 occurrence. Sui group introduced mismatches into crRNAs to improve allele discrimination, achieving the detection of SARS-CoV-2 *Spike* T478K, D614G, P681R, and P681H mutants in clinical samples with 70-78 % sensitivity and 100 % specificity (Fig. 6E) (Zhang *et al.* 2022b). And they further integrated an automatic digital microfluid into CRISPR-LAMP platform to realize labor-free and contamination-free detection. To meet SARS-CoV-2 diagnostics that are easy to use outside of centralized clinical laboratories, Myhrvold and colleagues reported one-pot SHERLOCK assay that allowed for RNA-extraction-free and isothermal POCT of SARS-CoV-2 and its VOCs, named SHINEv.2 (streamlined highlighting of infections to navigate epidemics, version 2). SHINEv.2 leveraged lyophilised reagents and rapid sample lysis at ambient temperature to eliminate the multiple heating steps and cold temperature storage requirement that SHINEv.1 suffered from (Fig. 6F) (Arizti-Sanz *et al.* 2022; Arizti-Sanz *et al.* 2020). SHINEv.2 enabled visual SARS-CoV-2 detection with 100% specificity and 90.5% sensitivity, and discrimination of SARS-CoV-2 VOCs including Alpha, Beta, Gamma, Delta and Omicron using LFA technology at body heat. Smartphones, integrating imageability, data analyzability, interactivity and potability can facilitate POCT. Ma *et al.* developed a AuNPs-based Cas12 colorimetric biosensing for POC diagnosis of SARS-CoV-2 using smartphone readout (Ma *et al.*

2022). It can detect 1 copy/μL pseudoviruses within 90 min, and provided 100% agreement with the qPCR test.

Compared with the gene testing, viral antigen detection using specific aptamer can reflect the viral infectivity and reduce contamination risk from enzymatic nucleic acid amplification. Zhang group reported a label-free and amplification-free electrochemical sensor employing aptamer to specifically recognize SARS-CoV-2 particles and Cas12a system to perform signal amplification (Fig. 6G) (Liu *et al.* 2022b). The competitive binding of specific aptamer to the nucleocapsid protein of SARS-CoV-2 released the activator of Cas12a, resulting significant signal change in impedance. This assay could complete the detection in 40 min without complex pretreatment process, and allowed a LOD of 0.077 ng nucleocapsid proteins per milliliter.

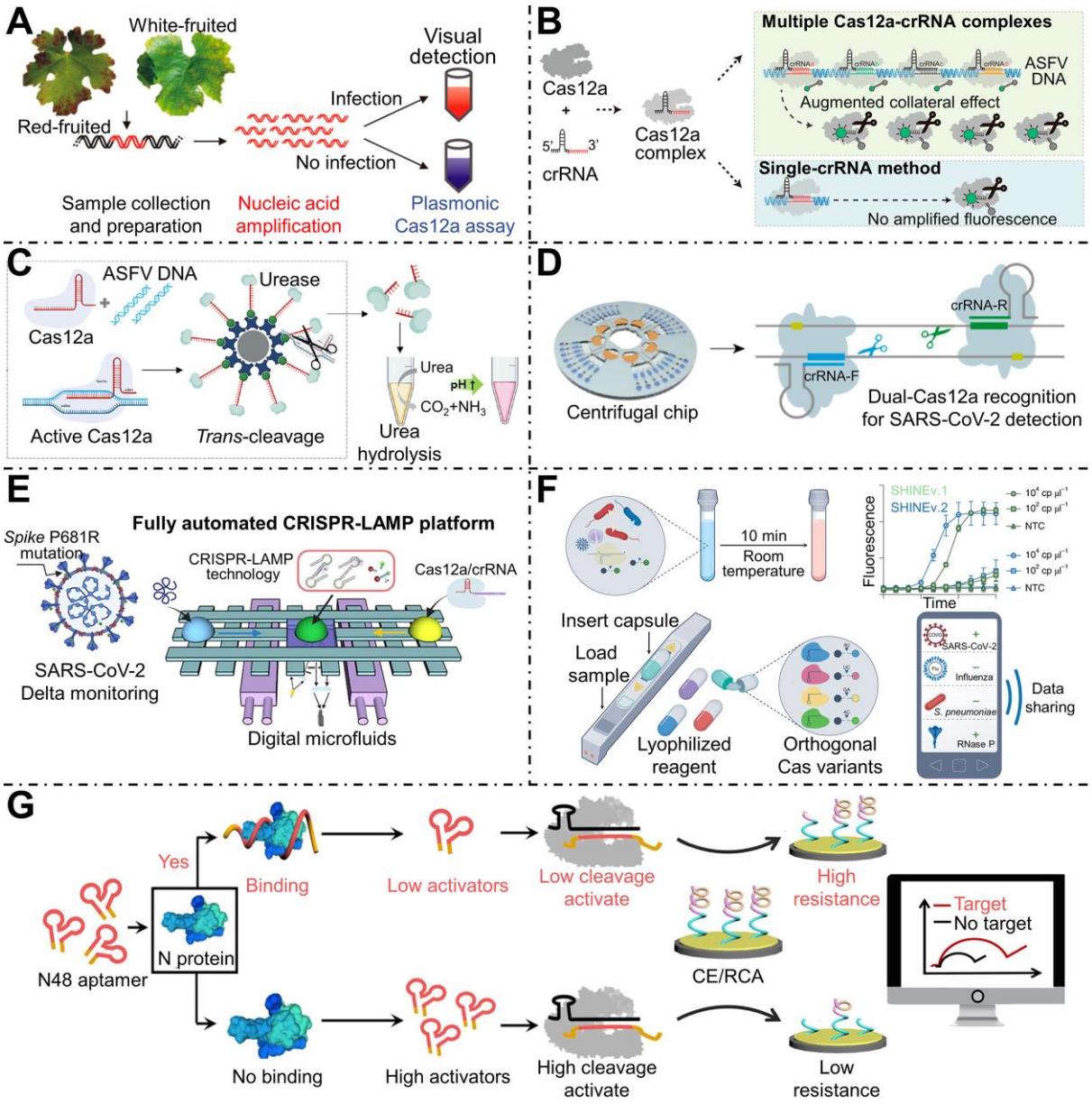


Fig. 6. CRISPR-based biosensors for detecting pathogenic virus. (A) AuNPs-based Cas12a assay for detection GRBV (Li *et al.* 2019c). Using DNA functionalized gold nanoparticles as the reporter of Cas12a cleavage enabled visual POCT diagnosis. (B) Multiple Cas12a/crRNA recognition for preamplification-free detection of ASFV (Zeng *et al.* 2022). Multiple Cas12a/crRNA recognition showed ~64-fold improved sensitivity than that using single crRNA for detecting ASFV. (C) Urease-catalyzed hydrolytic Cas12a assay for detection of ASFV (Ki *et al.* 2022). Using urease-catalyzed urea hydrolysis as reporter of Cas12a cleavage enabled visual POCT diagnosis. (D) Dual-Cas12a

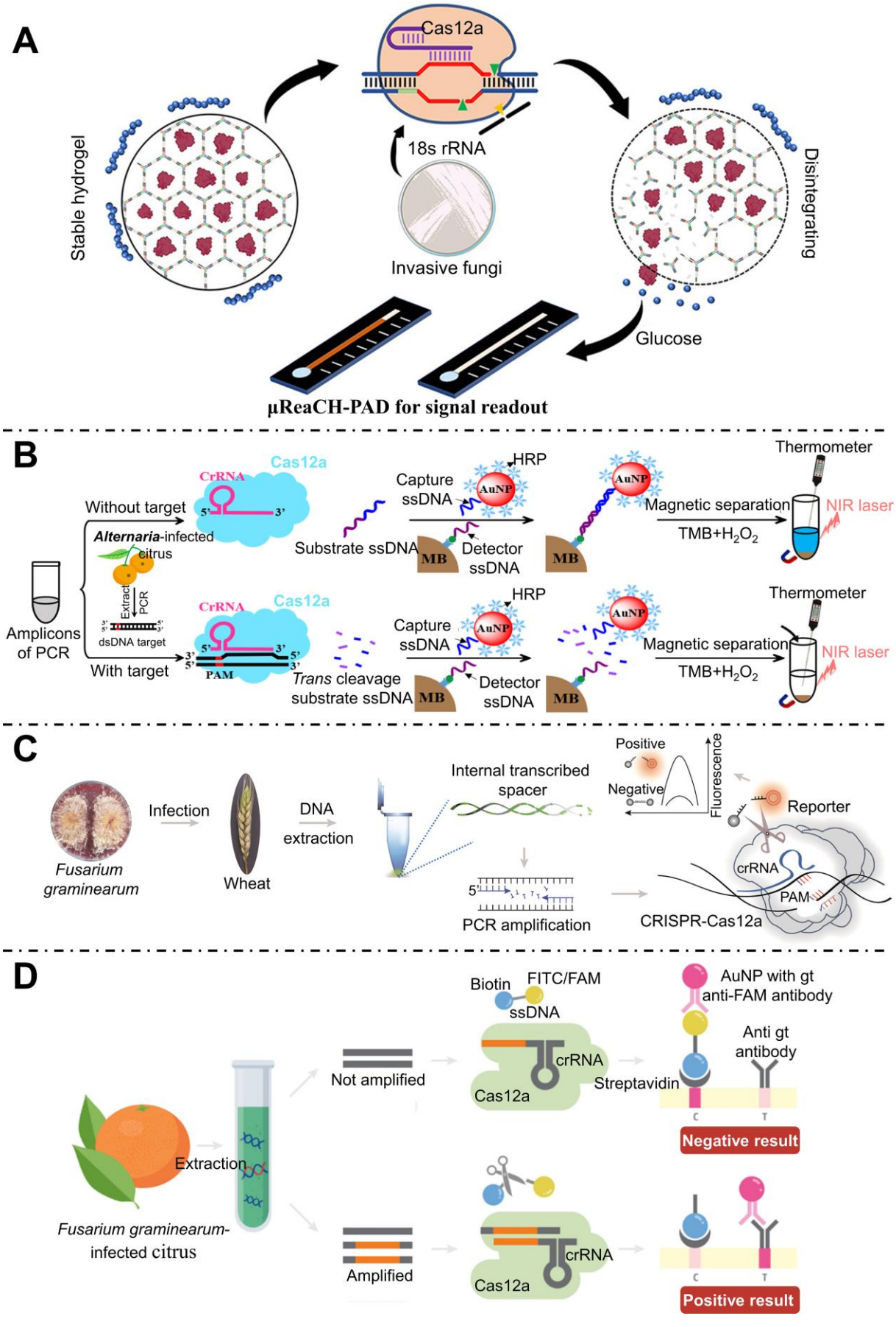
recognition for highly specific and sensitive detection of SARS-CoV-2 (Chen *et al.* 2022). Design of "sample-to-answer" microfluidic enabled automatically POC sensing. (E) CRISPR-LAMP platform with artificial mismatches for detection of SARS-CoV-2 VOCs (Zhang *et al.* 2022b). Use of digital microfluids enabled fully automated POCT diagnosis. (F) One-pot SHERLOCK assay (SHINE) for RNA-extraction-free and isothermal POCT diagnosis of SARS-CoV-2 and its VOCs (Arizti-Sanz *et al.* 2022). SHINEv.2 integrated lyophilised reagents and rapid sample lysis at ambient temperature, rendering it more feasible for POCT diagnosis than SHINEv.1. (G) Aptamer-based Cas12a assay for label-free detection of SARS-CoV-2 particles (Liu *et al.* 2022b). Use of aptamer enabled and RNA-extraction-free sensing of SRAS-CoV-2.

4.3. Fungi

Invasive fungal diseases have become a significant problem, not only causing severe loss of crop yields, but directly affecting human health. The commonly used clinical diagnostic culture-based method shows high accuracy but suffers from complicated operation and time-consumption, hampering the in-field testing (Safavieh *et al.* 2017). Another widely used method is morphology scanning, however, it cannot distinguish symptoms caused by various pathogens (Singha *et al.* 2016). Xu and colleagues developed a hydrogel paper-based analytical device using microfluidic ruler-readout and Cas12a-responsing for POCT diagnosis of invasive fungi (Fig. 7A) (Huang *et al.* 2021). Cas12a/crRNA was responsible for specifically targeting the genus-conserved 18s rRNA, subsequently using enzyme-caged DNA hydrogel for signal amplification and transduction, and microfluidic chips for visual quantification by naked eye. This assay can visually identify representative human fungal pathogens, *Candida* and *Aspergillus*,

as low as 10 CFU/mL. In addition, Liu *et al.* developed a Cas12a-based photothermal assay for POCT diagnosis. The assay involved only a thermometer, eliminating the need for cumbersome and expensive instruments traditionally used in morphological analysis and gene-sequencing. In the photothermal detection of *Alternaria* species using HRP-TMB reporter, the causative agents for citrus brown spot, this platform can detect target fungal genes as low as 1.5 pM in citrus, tomato, and apple samples (Fig. 7B) (Liu *et al.* 2022c).

Control of pathogenic fungal diseases in cereal crops requires reliable early diagnostic tools. As the primary causative agent of Fusarium head blight which is widespread in wheat-growing region, *Fusarium graminearum* (*F. graminearum*) may lead to severe yield reduction and generation of varieties of mycotoxins, contaminating food (Duan *et al.* 2014; Zhou *et al.* 2019). Classical PCR-coupled Cas12a-based nucleic acid assay was competent for the early diagnosis of scab of wheat caused by *F. graminearum* (Fig. 7C) (Mu *et al.* 2022). The assay allowed for highly specific discrimination of *F. graminearum* species and sensitive detection as low as 1 fg/μL total DNA, and it particularly can diagnose a 4-day infection of *F. graminearum*. Furthermore, fungal infection is a considerable problem in citrus, causing a significant drop in output quality. Shin *et al.* used a RPA-based Cas12a assay aided by LFA to achieve POC diagnosis of citrus scab (Fig. 7D) (Shin *et al.* 2021). The whole detection could be carried out within 1 h, and the LFA readout showed a high sensitivity down to 1 fg which allowed for robust detection even using crude DNA extracted from infected citrus tissue.


A
Cas12a
18s rRNA
Invasive fungi
Stable hydrogel
Disintegrating
Glucose
μReaCH-PAD for signal readout
B
Cas12a
CrRNA
Without target
Alternaria-infected citrus
Extract
PCR
dsDNA target
With target
PAM
Amplicons of PCR
Capture ssDNA
HRP
AuNP
Substrate ssDNA
MB
Detector ssDNA
Magnetic separation
TMB+H2O2
Thermometer
NIR laser
Trans cleavage substrate ssDNA
C
Infection
Fusarium graminearum
Wheat
DNA extraction
Internal transcribed spacer
PCR amplification
Positive
Negative
Fluorescence
Reporter
crRNA
PAM
CRISPR-Cas12a
D
Biotin
FITC/FAM
ssDNA
AuNP with gt anti-FAM antibody
Anti gt antibody
Streptavidin
Not amplified
Extraction
Fusarium graminearum-infected citrus
Amplified
Negative result
Positive result

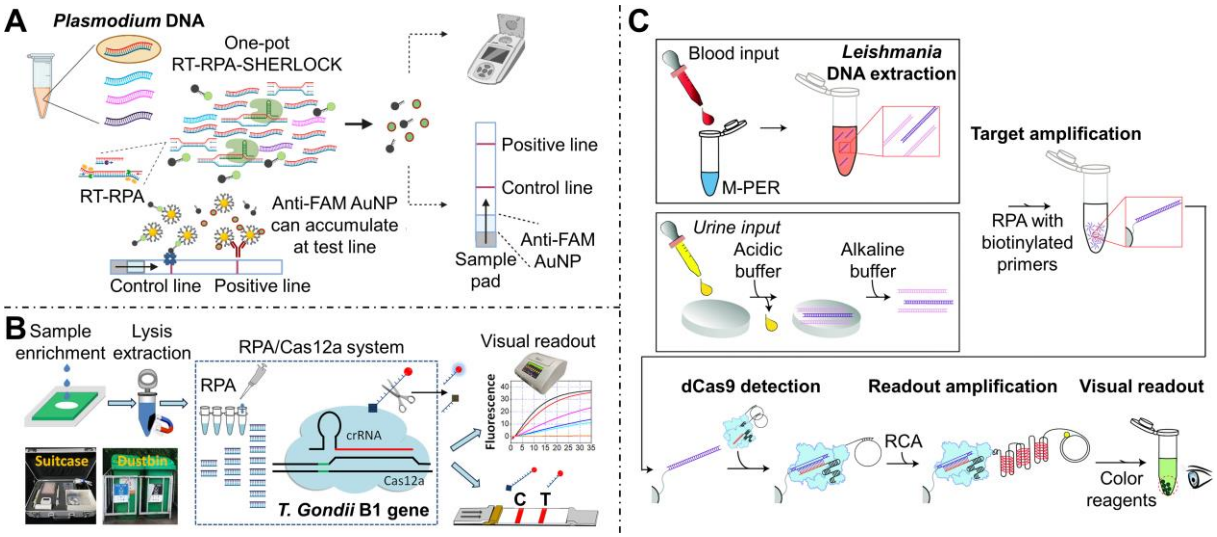
593

Fig. 7. CRISPR-based biosensors for detecting pathogenic fungi. (A) Hydrogel paper-based Cas12a assay for detection of invasive fungi (*Candida* and *Aspergillus*) (Huang *et al.* 2021). Use of microfluidic ruler-readout enabled visual POC sensing by naked eyes. (B) Photothermal Cas12a assay for detection of *Alternaria* species in fruit (Liu *et al.* 2022c). Use of HRP-TMB reporter and thermometer enabled photothermal POCT diagnosis. (C) PCR-coupled Cas12a-based nucleic acid assay for detection of *F. graminearum* infection in wheat (Mu *et al.* 2022). This assay can diagnose a 4-day *F. graminearum* infection. (D) Cas12a-based RPA-assay for detection of *F. graminearum* infection in citrus (Shin *et al.* 2021). Use of LFA enabled POC diagnosis of citrus scab.

4.4. Parasites

Parasitic helminths are the causative agents of devastating chronic diseases, posing a major threat to public health and economic development in tropical and subtropical low-income communities in developing countries. Developing advanced parasites-diagnostic platforms compatible for use in resource-constrained settings is conducive to achieve eradication of parasitic infections. It is reported that SHERLOCK platform enabled sensitive detection and differentiation of the malaria-causative *Plasmodium* species including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* (Fig. 8A) (Lee *et al.* 2020). The platform particularly presented a 10-min rapid extraction protocol for parasites, assisting to achieve one-pot and isothermal detection. And the detection limit (0.36-2.4 parasites per microliter blood) for plasmodium species met the requirement (below two parasites per microliter blood) that was suggested by the WHO. Except for malaria infection, *Toxoplasma gondii* (*T. gondii*)-causing toxoplasmosis is an important zoonotic disease. *T. gondii* is an

opportunistic pathogen capable of infecting nearly 1/3 of human populations around the world (Zhou *et al.* 2011). Wang group utilized the RPA-Cas12a-based nucleic acid assay to detect *T. gondii* (Fig. 8B) (Lei *et al.* 2022). They designed a glass microfiber filter device for sample enrichment and lysis extraction, enabling nucleic acid extraction of low-concentration of *T. gondii*. Sample enrichment and signal amplification simultaneously contributed to a high sensitivity of the assay that yielded a LOD of 3.3 copies/μL. Employing fluorometer and LFA strip can realize the visual POC detection, benefiting the assay to be used in remote areas. Leishmaniasis is one of the most neglected tropical diseases around the world. Gao *et al.* recently developed a G-quadruplex-substrated Cas12a assay achieved label-free detection of *Leishmania donovani* with single-cell sensitivity (Gao *et al.* 2022). This assay can diagnose *Leishmania* infection in mice nearly 2 weeks earlier than the conventional parasitological analysis. dCas9 coupling with RCA can realize sensitive POCT diagnosis, Dekker et al used this strategy to isothermally detect the genus *Leishmania* with a sensitivity of 1 zeptomole (Fig. 8C) (Bengtson *et al.* 2022). The G-quadruplexes produced by RCA had peroxidase activity capable of catalyzing the color change of $ABTS^{2-}$ in the presence of hemin. This colorimetric readout achieved a visual detection with the naked eye.



634

635 **Fig. 8. CRISPR-based biosensors for detecting parasites.** (A) One-pot RT-RPA-SHERLOCK

636 assay for detection of the malaria-causative *Plasmodium* species (Lee *et al.* 2020). (B) Cas12a-based

637 RPA assay for detecting *T. gondii* (Lei *et al.* 2022). Use of a glass microfiber filter device for sample

638 enrichment and lysis extraction, enabling nucleic acid extraction of low-concentration of *T. gondii*.

639 (C) dCas9 coupling with RCA for the detection of *Leishmania* (Bengtson *et al.* 2022). Use of G-

640 quadruplexes DNAzyme enabled a visual detection.

641 **Table 1. Overview of CRISPR-based biosensors for pathogen detection.**

Cas protein	Analyte type	Amplification method	Auxiliary tool	Target	Assaying time	Sensitivity	Samples	Ref.
Cas9	Bacteria	RCA	MOF (UiO66)	*E. coli* O157:H7	120 min	10 CFU/mL	Spring water, skim milk and orange juice	(Sun *et al.* 2020b)
	Bacteria	-	Magnetic separation	MRSA	90 min	10 CFU/mL	-	(Guk *et al.* 2017)
	Fungus	RT-RPA	LFA strip	*Puccinia striiformis* f. sp. *tritici* and *Magnaporthe oryzae Triticum*	60 min	-	Infected wheat plants	(Sánchez *et al.* 2022)
	Parasite	RPA and RCA	G- quadruplex	*Leishmania*	-	1 zeptomole	Blood	(Bengtson *et al.* 2022)
Cas12a	Bacteria	RPA	Magnetic separation and aptamer	MRSA	95 min	8 CFU/mL	Clinical samples	(Wei *et al.* 2022a)
	Bacteria	RPA	Microfluidic paper	*S. typhimurium*	45 min	3-4 CFU/mL	Milk and meat	(Zhuang *et al.* 2022)
	Bacteria	-	Aptamer and biotin-streptavidin	*Acinetobacter baumannii*	30 min	3 CFU/mL	-	(Li *et al.* 2020)
	Bacteria	PCR	AuNPs	*Salmonella*	60 min	1 CFU/mL	Milk	(Ma *et al.* 2021a)

Bacteria	LAMP	G^- Quadruplex	*S. enterica*	50 min	20 CFU	*In Vivo* colonization in chickens	(Xia *et al.* 2021)
Bacteria	RAA	Gold electrode	*L. monocytogenes*	10 min	26 CFU/mL	*Flammulina velutipes*	(Li *et al.* 2021b)
Bacteria	RCA	Antibody magnetic beads	*E. coli O157:H7*	100 min	10 CFU/mL	Milk	(Chen *et al.* 2021)
Bacteria	RPA	AuNPs, magnetic separation	*S. typhimurium*	60 min	1 CFU/mL	Milk	(Cai *et al.* 2021)
Bacteria	PCR/RAA	LFA strip	*S. aureus*	60 min	1 CFU/reaction	Milk	(Qian *et al.* 2022)
Bacteria	Isothermal amplification	Aptamer and Au electrode	*E. coli O157:H7*	120 min	19 CFU/mL	Milk	(Bu *et al.* 2021)
Bacteria	RPA	-	*Enterocytozoon hepatopenaei*	40 min	10 gene copies per reaction	Shrimp	(Wang *et al.* 2023)
Bacteria	HCR	All-fiber	*E. coli* O157:H7	50 min	17.4 CFU/mL	Tap water and secondary sedimentation tank effluent	(Song *et al.* 2023)
Bacteria	LAMP	Membrane filter	*E. coli O157:H7*	70 min	1.22 CFU/mL	Romaine Lettuce	(Lee and Oh 2022)
Bacteria	RAA	LFA strip	*S. aureus*	70 min	540 CFU/mL	Beef, pork, tomato, coriander and lettuce	(Zhou *et al.* 2022)
Bacteria	NEAA	-	*Salmonella*	20 min	80 CFU/mL	Egg	(Bai *et al.* 2022)
Bacteria	NASBA	-	*S. enterica*	150 min	1 CFU	Egg shell, beef, pork, duck, chicken, mutton and tap water	(Xue *et al.* 2022)

Bacteria	-	Magnetic separation	MRSA	100 min	16 CFU/mL and 0.01 % SNP	Eggs, milk and pork	(Wei *et al.* 2023)
Bacteria	LAMP	Silver nanoclusters	*S. typhimurium*	55 min	1 CFU/mL	Orange juice and eggs	(Ma *et al.* 2023)
Bacteria	PCR or RPA	-	Drug-resistant *Salmonella*	45 min	1 CFU/mL	Milk and skim milk powder	(Fu *et al.* 2022)
Bacteria	ARMS-PCR	-	*S. enterica*	60 min	~0.5 % SNP	-	(Yang *et al.* 2022b)
Virus	PCR	AuNPs	GRBV	30 min	40 pM	Grapevine	(Li *et al.* 2019c)
Virus	-	Multiple crRNAs	ASFV	90 min	1 pM	-	(Zeng *et al.* 2022)
Virus	-	Magnetic beads and urease	ASFV	60 min	50 pM	Porcine clinical tissue	(Ki *et al.* 2022)
Virus	RAA		African swine fever virus	60 min	10 aM	Pig blood	(Bai *et al.* 2019)
Virus	PCR or RPA	-	WSSV	60 min	200 copies per reaction	Shrimp	(Chaijaras phong *et al.* 2019)
Virus	RT-RPA	LFA strip	Norovirus	40 min	965 copies/mL	Clinical samples from patients with gastroenteritis	(Qian *et al.* 2021)
Virus	RT-RPA		SARS-CoV-2	45 min	10 copies	Clinical samples	(Meng *et al.* 2021)
Virus	RAA	Microfluidics	SARS-CoV-2	30 min	1 copy/μL	Throat swab	(Chen *et al.* 2022)
Virus	LAMP	Microfluidics	SARS-CoV-2	45-75 min	100 copies/μL	Nasopharyngeal swab	(Zhang *et al.* 2022b)

Virus	RT-PCR	AuNPs	SARS-CoV-2	90 min	1 copy/μL	Throat swab	(Ma *et al.* 2022)
Virus/viroids	RT-RPA	AuNPs	Multiple prevalent RNA viruses/viroids in apple	30 min	250 or 2500 viral copies per reaction	Crude apple leaf	(Jiao *et al.* 2021)
Fungus	RAA	Microfluidic chips	*Candida* and *Aspergillus*	90 min	4.90 and 4.13 CFU/mL	Ascites, wound discharge, urine, sputum, blood	(Huang *et al.* 2021)
Fungus	PCR	Magnetic beads, AuNPs and thermometer	*Alternaria*	110 min	1.5 pM	Citrus, apple and tomato	(Liu *et al.* 2022c)
Fungus	PCR	-	*F. graminearum*	120 min	1 fg/μL	Wheat	(Mu *et al.* 2022)
Fungus	RPA	LFA strip	*Elsinoë fawcettii*	150 min	1 fg	Citrus	(Shin *et al.* 2021)
Parasite	RT-RPA	LFA strip	*Plasmodium*	70 min	<2 parasites per microliter blood	Blood	(Lee *et al.* 2020)
Parasite	RPA	LFA strip	*T. gondii*	55 min	3.3 copies/μL	Heavily polluted landfill leachate	(Lei *et al.* 2022)
Parasite	RAA	-	*T. gondii*	70 min	1 fM	Soil	(Ma *et al.* 2021b)
Parasite	RPA	LFA strip	*Cryptosporidium parvum*	90 min	1 copy of GP60 gene and 10 oocysts/g	Human and cattle fecal	(Yu *et al.* 2021)
Parasite	RPA	G-quadruplex	*Leishmania donovani*	120 min	3.1 parasites	Infected mice spleen samples	(Gao *et al.* 2022)

Cas13a	Bacteria	Transcription	Aptamer	*Salmonella* Enteritidis	60 min	1 CFU	Milk and serum	(Shen *et al.* 2020)
	Bacteria	PCR and transcription	-	*S. typhimurium*	120 min	1 CFU/mL	Milk and juice	(Gao *et al.* 2021)
	Bacteria	RAA	Microfluidic chip	*Listeria*	60 min	12.1-72.1 aM	*Flammulina velutipes*	(Xiang *et al.* 2022)
	Bacteria	-	Light-up RNA aptamer	*Bacillus cereus*	20 min	10 CFU	Rice and milk	(Zhang *et al.* 2021)
	Virus	Transcription	Light-up RNA aptamer	SARS-CoV-2	180 min	82 copies	Food packages, seafood and throat swabs	(Wang *et al.* 2021c)
	Virus	RT-RPA and transcription	LFA strip	SARS-CoV-2	90 min	200 copies/μL	Nasopharyngeal swabs	(Arizti-Sanz *et al.* 2022)
	Virus	RT-PCR	Microfluidic chip	SARS-CoV-2	180 min	1000 copies/μL	Nasopharyngeal swabs	(Welch *et al.* 2022)
	Virus	-	-	SARS-CoV-2	35 min	84 aM	Saliva samples	(Yang *et al.* 2022c)
	Parasite	RPA and transcription	-	*Plasmodium*	210 min	2.5-18.8 parasites/μL	Blood	(Cunningham *et al.* 2021)
Cas14a	Bacteria	RT-asymmetric PCR	-	*Eberthella typhi* and *Streptococcus pyogenes*	60 min	10^4 CFU/mL	Milk	(Ge *et al.* 2021)
	Bacteria	RT-PCR	Magnetic bead	*E. coli*	60 min	1 CFU/mL	Clinical blood	(Song *et al.* 2021)

	Bacteria	-	Aptamer	*S. aureus*	210 min	400 CFU/mL	Coconut water and milk	(Wei *et al.* 2022b)

642

5. Concluding remarks and perspectives

Analytical tools for pathogenic biosafety have advanced rapidly. Among them, CRISPR-based biosensors, derived from synthetic biology tools, are of growing interest due to their specific recognition capability, high cleavage activity and easy programmability. As shown in Table 1, these assays have evolved into multitudes of analytical techniques for rapid, sensitive and on-site detection of different pathogens associated with biosafety. Particularly, the COVID pandemic accelerate the development of CRISPR-based biosensors for POCT application. However, several challenges remain in applying CRISPR-based biosensors for practical and commercialized use. First, in field diagnostics, the complex matrix derived from samples may inference the amplification and signal yield, so pretreatments such as extraction and purification are usually needed. Development of rapid and convenient extraction devices, such as microneedles (Paul *et al.* 2019) and microfiber filters (Lei *et al.* 2022), can facilitate the CRISPR-based biosensors for on-site detection. Second, the emerging pathogenic variants, particularly those associated with single-nucleotide mutation, highlight the demand for nucleic acid assays with single-nucleotide resolution. The design of chemical modification and artificial mismatches in gRNA (Rozners 2022; Yang *et al.* 2022a), as well as engineered Cas proteins (such as split Cas12a) (Nihongaki *et al.* 2019) hold the potential to improve the assaying specificity to discriminate mutations in pathogenic variants. Third, preamplification makes the detection process complicated, time-consuming, and possibly induces false-positive caused by contamination. Strategies such as the use of multiplex Cas effectors, cascade CRISPR

assays (Gootenberg *et al.* 2018; Sha *et al.* 2021; Shi *et al.* 2021) and droplet microfluidic platforms (Sun *et al.* 2020a; Tian *et al.* 2021) may enable nucleic acid preamplification-free detection with femtomolar sensitivity. Fourth, pathogenic variants involve multiple mutations and infections may be caused by different pathogens, thus highlighting the development of multiplexing assays. Different targeting Cas effectors and reporting approaches can be exploited to meet the multiplex detection, where the class 1 CRISPR system (Mohanraju *et al.* 2022) and protein reporter-based CRISPR system (e.g., Craspase) (Hu *et al.* 2022) could be candidates. In addition, it is necessary to seek and develop new signal transduction elements that can be combined with CRISPR system for non-targeted screening of pathogens, as risky pathogens are rapidly emerging. The development of enzyme engineering, nanotechnology, and microfluidic techniques would facilitate the CRISPR system to serve as critical tools for pathogenic biosafety monitoring.

CRediT author contribution statement

Hao Yang: Conceptualization, Writing – original draft, Writing – review & editing. **Rodrigo Ledesma-Amaro**: Writing – review & editing. **Hong Gao**: Writing – review & editing. **Yao Ren**: Conceptualization, Supervision, Writing – review & editing. **Ruijie Deng**: Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of competing interest

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

Acknowledgement

This work was financially supported by National Natural Science Foundation of China (No. 22074100), the Young Elite Scientist Sponsorship Program by CAST (no.YESS20200036), the Green Manufacturing Project of Ministry of Industry and Information Technology of China.

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The authors declared that they have no conflicts of interest to this work.

CRISPR-based biosensors for pathogenic biosafety

Hao Yang[1], Rodrigo Ledesma-Amaro[2], Hong Gao[1], Yao Ren[1,*], Ruijie Deng[1,*]

[1] College of Biomass Science and Engineering, Healthy Food Evaluation Research Center, Sichuan University, Chengdu 610065, China

[2] Department of Bioengineering, Imperial College Centre for Synthetic Biology, Imperial College London, London SW7 2AZ, UK

Corresponding author

Ruijie Deng, e-mail: reny@scu.edu.cn; drj17@scu.edu.cn

Abstract:

Pathogenic biosafety is a worldwide concern. Tools for analyzing pathogenic biosafety, that are precise, rapid and field-deployable, are highly demanded. Recently developed biotechnological tools, especially those utilizing CRISPR/Cas systems which can couple with nanotechnologies, have enormous potential to achieve point-of-care (POC) testing for pathogen infection. In this review, we first introduce the working principle of class II CRISPR/Cas system for detecting nucleic acid and non-nucleic acid biomarkers, and highlight the molecular assays that leverage CRISPR technologies for POC detection. We summarize the application of CRISPR tools in detecting pathogens, including pathogenic bacteria, viruses, fungi and parasites and their variants, and highlight the profiling of pathogens' genotypes or phenotypes, such as the viability, and drug-resistance. In addition, we discuss the challenges and opportunities of CRISPR-based biosensors in pathogenic biosafety analysis.

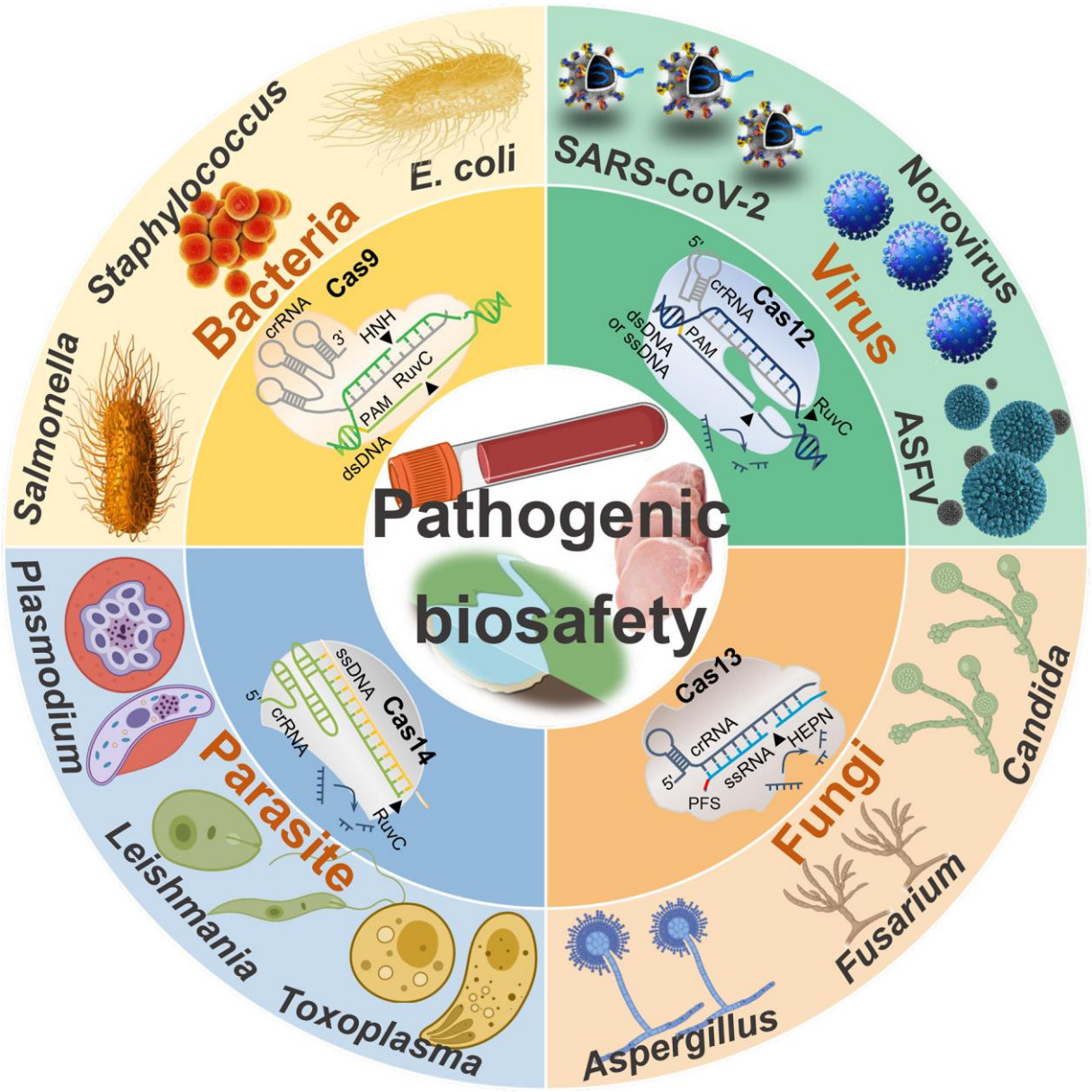
Keywords: point-of-care; SARS-CoV-2 variants; drug-resistance; fungi; parasites; viable bacteria

1. Introduction

Pathogenic biosafety concerns have been growing in parallel to human progress and development (Jiang *et al.* 2021; Xiao *et al.* 2021). With economic globalization, countries over the world are interdependent on their supply and trade contacts, thus easily resulting in biosafety issue such as pathogen-caused. Pathogens, mainly including bacteria, viruses, fungi and parasites, are widely distributed in food and environment, and can cause both diseases and economic losses (Fraiture *et al.* 2017; Liu *et al.* 2021b; Shin *et al.* 2022; Yang *et al.* 2020; Yang *et al.* 2021b). For example, the Centers for Disease Control and Prevention (CDC) reported that human salmonellosis may cause approximately ~450 deaths out of ~1.2 million diseases per year in the United States, which often accompanied by clinical systemic symptoms with 8-72 h, such as bacteremia, fever and severe diarrhea (Farka *et al.* 2016; Yang *et al.* 2022b). The World Health Organisation (WHO) statistics showed that the current SARS-CoV-2 outbreak around the world has infected nearly 649 million people, of which over 6.65 million have died, as of December 20, 2022 (WHO 2022a). In 2020, there were an estimated 241 million confirmed cases and 627 thousand deaths from malaria globally (WHO 2022b). Pathogenic propagation inhibition and elimination require reliable and widely available detection tools. Most of the current methods to detect pathogens rely on culture and colony counting, quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA) (Xiong *et al.* 2020a). However, their high-cost or long turnaround time severely limits their applications, particularly in resource-constrained settings (Harshit *et al.* 2017; Li *et al.* 2019a; Randazzo *et al.* 2018; Xiong *et al.* 2020a).

The key to improve speed, accuracy and specificity of analytical technologies for pathogenic biosafety lies on developing new recognition and signal transduction patterns. A promising technology is the use of clustered regularly interspaced short palindromic repeat (CRISPR)-associated (CRISPR/Cas) systems. Because of its remarkable target sequence specificity, flexibility of design and ease of use, a myriad of biosensors based on CRISPR systems have been recently developed for POCT diagnosis, food and environmental safety analysis (Dai *et al.* 2020; Freije and Sabeti 2021; Li *et al.* 2019b; Li *et al.* 2022a; Li *et al.* 2022b; Yin *et al.* 2021b).

In this article, we first reviewed the mechanism and properties of class II CRISPR/Cas system, and discussed how CRISPR tools (Cas9, Cas12 and Cas13) have been leveraged for molecular assays for detecting nucleic acid and non-nucleic acid biomarkers (Fig. 1) (Chen *et al.* 2018; Gootenberg *et al.* 2018; Gootenberg *et al.* 2017; Harrington *et al.* 2018b; Ishino *et al.* 1987; Lee *et al.* 2020; Zhu *et al.* 2014). We then highlighted the strategies for CRISPR-based visual biosensing that capable of POCT diagnosis, such as the readouts using lateral-flow assay (LFA) papers and smartphones. We next summarized the CRISPR-based biosensing applications in pathogenic biosafety analysis including bacteria, viruses, fungi, parasites and their variants, and highlighted the analysis of pathogens' genotypes or phenotypes, such as viability and drug-resistance. Finally, challenges and opportunities of the CRISPR-based biotechnologies for pathogenic biosafety are also discussed.



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66 **Fig. 1. Schematic diagram of advances of CRISPR-based biosensors for pathogenic biosafety.**

67 CRISPR/Cas systems include Cas9, Cas12, Cas13 and Cas14; pathogens include bacteria, viruses,

68 fungi, parasites and their variants.

69 **2. Principle of CRISPR-based biosensors**

70 Following the discovery of unusual sequences of repeated DNA in *E. coli* (Ishino *et*

71 *al.* 1987), researchers soon found their role in the adaptive immune system of about 87%

72 of archaea and 47% of bacteria (Mojica *et al.* 2005). The type of sequences, known as

CRISPR, recognizes exogenous nucleic acids and subsequently cut with Cas endonucleases (Barrangou and Marraffini 2014; Jansen *et al.* 2002; Nouri *et al.* 2021; Stout *et al.* 2017). Two essential components, a CRISPR RNA (crRNA) and Cas protein, underlie the foundation of the CRISPR/Cas systems. The crRNA guides Cas endonuclease toward a specific DNA or RNA sequence of interest via complementary hybridization, triggering the cleavage activity on target nucleic acids (*cis*-) or non-target collateral ones (*trans*-) (Eş *et al.* 2019). The target regions in class II CRISPR systems, which widely used in applications of pathogen monitoring, are restricted to the vicinity of a protospacer adjacent motif (PAM) or protospacer flanking sequence (PFS). Cas effectors in class II CRISPR systems are widely used for molecular analysis and can be divided into two categories: DNA- and RNA-targeting enzymes. The DNA-targeting enzymes encompass Cas9 and Cas12 (Harrington *et al.* 2018a; Li *et al.* 2019d; Yin *et al.* 2020), both of which are limited to PAM motifs, while the RNA-targeting enzyme Cas13 needs PFS sequences (Gootenberg *et al.* 2017).

Activation of the target-dependent multiple-turnover *trans*-cleavage of Cas effectors via hybridization to a complementary target sequence enables CRISPR tools for a specific signal amplification of sequence recognition. Therefore, CRISPR/Cas system presents immense potential to be used for constructing biosensors due to its capacity for target recognition and signal amplification.

2.1. Nucleic acid biomarkers

Pathogenic contaminants can be identified through nucleic acid detection against their genomic DNA or RNA (Fig. 2A). In general, coupling CRISPR/Cas systems with

nucleic acid amplification strategies, like nucleic acid sequence-dependent amplification (NASBA) (Pardee *et al.* 2016), polymerase chain reaction (PCR) (Wang *et al.* 2021a), recombinase polymerase amplification (RPA) (Gootenberg *et al.* 2017), rolling circle amplification (RCA) (Yang *et al.* 2021a) and loop-mediated isothermal amplification (LAMP) (Broughton *et al.* 2020), can dramatically enhance the sensitivity and specificity of nucleic acid detection.

Cas9, including its variants, was the first CRISPR tool for constructing nucleic acid assays, targeting double-stranded DNA (dsDNA) (Bao *et al.* 2020; Pardee *et al.* 2016; Zhang *et al.* 2017; Zhou *et al.* 2018). NASBA-CRISPR cleavage (NASBACC), a Cas9-based strategy, combined toehold switches for the readout (Fig. 2B) (Pardee *et al.* 2016). A toehold trigger and Cas9-based specific cleavage were introduced into NASBA amplification. Cas9-mediated cleavage could be activated in the presence of a PAM sequence in the dsDNA produced from reverse transcription (RT), leading to a fragmented RNA without the toehold trigger. On the contrary, the NASBA-amplified full-length RNA contained the trigger, in the absence of the PAM, activated the toehold switch, as indicated by a colorimetric readout. The biosensing strategy yielded a high sensitivity (femtomolar range) and was capable of differentiating the arboviruses Dengue (DENV) from the Zika virus (ZIKV). The nCas9, with a single amino acid mutation in either the HNH (Cas9H840A) or RuvC (Cas9D10A) domain, can also play a nickase role; in the first case, it retains only the RuvC activity and therefore it only nicks target strand, and in the other case, only the non-target strand would be nicked (Gasiunas *et al.* 2012; Ran *et al.* 2013; Zhou *et al.* 2018). In 2018, a nCas9-based method

named CRISDA (CRISPR-nCas9-triggered nicking endonuclease-mediated strand displacement amplification) that enabled exponential strand displacement amplification (E-SDA) without heating steps was reported (Fig. 2D). 3' overhang contained primers bind to the nicked strand, E-SDA was then started with the aid of a DNA polymerase and ssDNA binding proteins (SSBs). CRISDA exhibited high sensitivity in the attomolar range and specificity of single-nucleotide discrimination (Zhou *et al.* 2018).

A well-known tool, which utilized for Cas12 enzymes for nucleic acid detection was named DNA endonuclease-targeted CRISPR *trans* reporter (DETECTR) (Chen *et al.* 2018) (Fig. 2C, bottom). Other DETECTR-derived techniques included Cas14a-DETECTR, which required complementarity in the seed region for ssDNA substrate recognition, and exhibited higher fidelity than Cas12a-DETECTR for DNA single-nucleotide polymorphism (SNP) discrimination (Harrington *et al.* 2018a). SARS-CoV-2 DETECTR achieved a short sample-to-result time of ~45 min that was markedly shorter than that using RT-qPCR (4 h), and presented a high sensitivity (10 copies per μL) when combined with LAMP preamplification (Broughton *et al.* 2020).

The RNA-targeting enzyme Cas13 has been combined with nucleic acid preamplification to form biosensing platforms called specific high-sensitivity enzymatic reporter unlocking (SHERLOCK), which allowed rapid and sensitive sensing of desired DNA or RNA sequences (Fig. 2C, top) (Gootenberg *et al.* 2017; Kellner *et al.* 2019). SHERLOCK could be used to determine ZIKV ssRNA with a limit of detection (LOD) of ~2 aM, which is 7 times more sensitive than Cas13a alone and enables SNP detection (Gootenberg *et al.* 2017). In addition, its sibling method called SHERLOCKv2 could

achieve a multiplexing test. Four Cas effectors, AsCas12a, LwaCas13a, CcaCas13b and PsmCas13b, were used to construct four independent signal channels for simultaneous multi-target detection (Gootenberg *et al.* 2018).

Nevertheless, target preamplification makes CRISPR-based biosensors time-consuming and susceptible to be interfered with sample matrices. The rational design of cascade CRISPR assays can enable preamplification-free sensing (Fig. 2E). For example, a cascade CRISPR/Cas assay (casCRISPR) which coupled Cas13a with Cas14a has been developed, allowing amplification-free, fM-sensitive and SNP-specific detection of RNA. Different versions of casCRISPR have been created. When comparing Cas13a-Cas12a (casCRISPR v2) with Cas13a-Cas14a (casCRISPR v1), it was shown that casCRISPR v1 performed better on the LOD of ~6.25 fM while it was ~100 fM for casCRISPR v2 (Sha *et al.* 2021). Furthermore, a hybrid CRISPR/Cas that fused Cas12a and Cas13a to a single protein enabled to simultaneously target DNA and RNA in a single tube. The hybrid CRISPR/Cas eliminated the use of multi-enzymes, which can address the drawbacks in the incompatible reaction conditions (Guk *et al.* 2023). CRISPR/Cas-only amplification network (CONAN) requiring no nucleic acid amplification can be achieved for the isothermally sensitive detection of genomic DNA (Shi *et al.* 2021). Rationally integrating Cas12a-crRNA with reporter into a switchable sgRNA achieved a positive feedback circuit-caused exponential dynamic, enabling an aM-sensitivity against the genomic DNA of hepatitis B virus. In addition, an auxiliary CRISPR-associated enzyme, termed Csm6, has attracted interest. Combining Csm6 with

Cas13a in a cascade increased the signal sensitivity for nucleic detection by ~3.5 times compared to that only using Cas13a (Gootenberg *et al.* 2018; Liu *et al.* 2021a).

The features of simplicity, programmability, compatibility, specificity and the capability in signal amplification make CRISPR/Cas tools outperform other natural or engineered molecular scissors, such as restriction endonucleases or deoxyribozymes (DNAzymes). Despite the advantages in dealing with nucleic acid targets, a predominant challenge for CRISPR-based assays is the fact that non-nucleic acid biomarkers are also critical in pathogenic biosafety. Confronting this challenge requires the development of signal transduction mechanisms that convert non-nucleic acid recognition into nucleic acid switch.

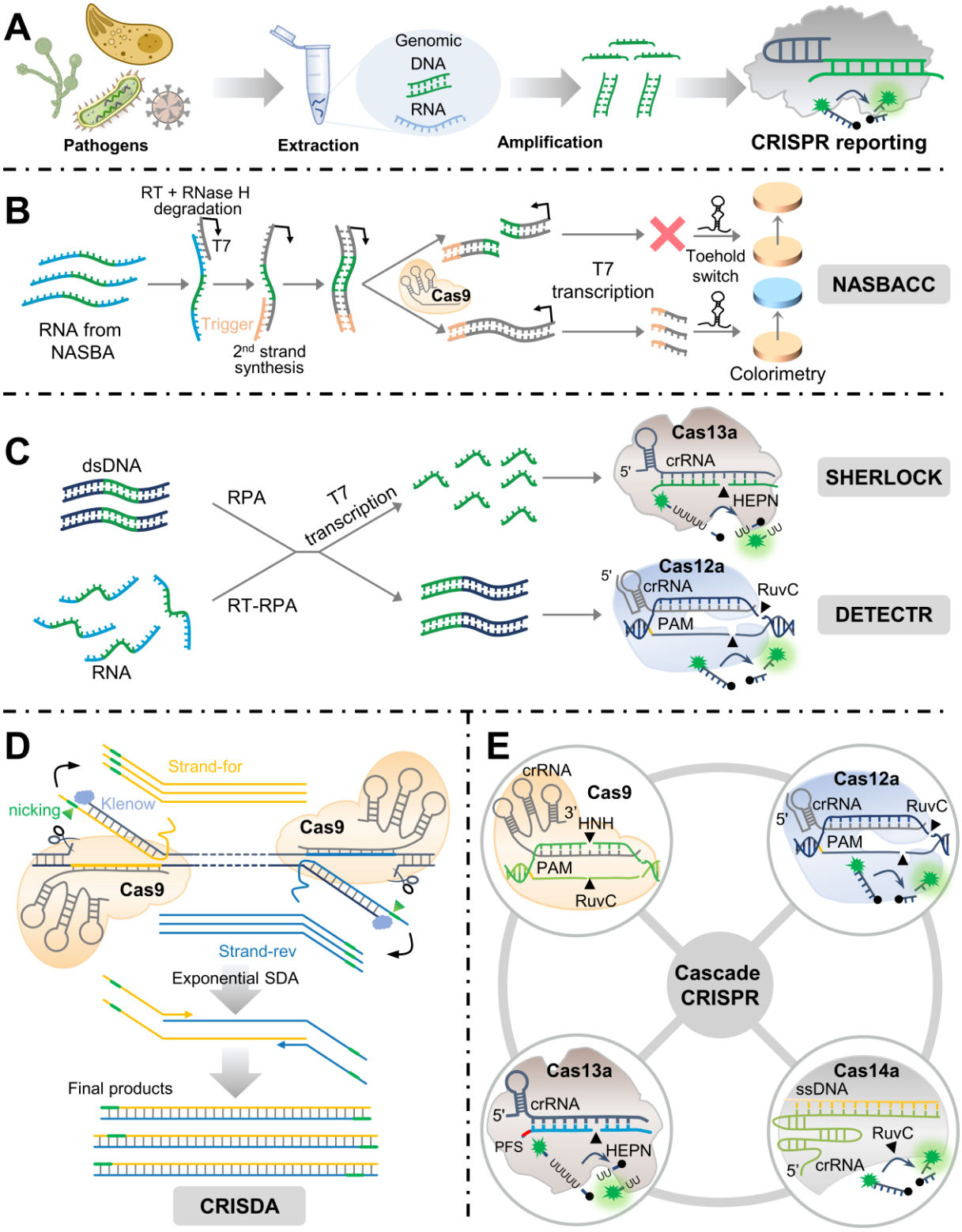


Fig. 2. CRISPR/Cas-based biosensors for pathogenic nucleic acid biomarkers. (**A**) Nucleic acid targets (genomic DNA or RNA) from pathogens need to be extracted, and being detected by CRISPR reporting. (**B**) NASBACC assay (Pardee *et al.* 2016). RNA targets from NASBA amplification

started with RT, then RNase H degraded the RNA from the RT-derived RNA/DNA hybrid. The binding of the complementary DNA to a primer that appended a trigger element for the toehold color sensor activates the 2nd DNA strand synthesis. Cas9-based cleavage truncated the dsDNA template that contained a PAM motif for T7 transcription, leading to no activation of toehold sensor, while producing a visible change without the PAM. (**C**) SHERLOCK and DETECTR assay (Chen *et al.* 2018; Gootenberg *et al.* 2017). RNA-targeting Cas13a, the amplified products needed to be T7-transcribed into RNA. The amplicons (DNA or RNA) bound to the guided-RNA to activate the Cas12a (used in DETECTR) or Cas13a (used in SHERLOCK) and subsequently triggered the fluorescent reporting. (**D**) CRISDA assay (Zhou *et al.* 2018). A pair of Cas9(H840A) enzymes as nickase were employed to recognize and nick each border of target DNA. A pair of nicking site-contained primers bound to the exposed non-target strand and trigger the exponential SDA amplification with the assistance of SSBs, generating a mass of expected final products (Strand-for and Strand-rev). (**E**) Cascade CRISPR assay (Sha *et al.* 2021; Shi *et al.* 2021). Combination of Cas9, Cas12a, Cas13a and Cas14a in cascade applications was expected to increase sensitivity without the need for preamplification.

2.2. Non-nucleic acid biomarkers

Multitudes of analytes related to pathogenic biosafety are non-nucleic acid targets, such as the entire pathogenic cells, specific antigens and antibodies, which cannot be directly detected by CRISPR-driven assays. However, tools for molecular recognition or signal transduction, such as antibodies, functional nucleic acids (aptamers or DNAzymes) and other molecular translators (Li *et al.* 2021a; Li *et al.* 2021c; Lin *et al.* 2019; Peng *et al.* 2018), enable CRISPR/Cas system to be used for detection of non-

196 nucleic acid biomarkers (Fig. 3A). Translators for metal ions or chemical analytes can
197 be artificially selected via an *in vitro* process (systematic evolution of ligands by
198 exponential enrichment, SELEX) (Deng *et al.* 2014; Peng *et al.* 2018).

199 CRISPR/Cas system can be combined with traditional ELISA platforms (Fig. 3B),
200 which may avoid complicated chemical modifications and laborious purification steps
201 for traditional ELISA (Acharya *et al.* 2013; Bruch *et al.* 2019). Li group described an
202 immunoassay based on aptamer-linked CRISPR/Cas12a for detecting non-nucleic acid
203 analytes (Li *et al.* 2021c). Antigen-aptamer complex as the linker was divided into two
204 parts, one of which for the capture module construction and another one for the aptamer-
205 linked CRISPR/Cas12a module. The platform yielded a higher sensitivity than ELISA
206 alone for protein (carcino-embryonic antigen), bacteria (*Escherichia coli* O157:H7, *E.*
207 *coli* O157:H7) and virus (norovirus) analysis.

208 Additionally, DNAzyme, a single-stranded DNA with high catalytic activity,
209 promises for bacteria detection when integrated with the CRISPR/Cas systems (Fig. 3C).
210 CIM (crude intracellular mixture) and CEM (crude extracellular mixture) could be used
211 for direct detection of bacteria (Ma *et al.* 2021c). DNAzymes were *in vitro* selected for
212 the recognition of *Clostridium difficile* (Shen *et al.* 2016), *E. coli* (Ali *et al.* 2011) and
213 *Helicobacter pylori* (Ali *et al.* 2019), have been selected *in vitro*. In the DNAzyme assay,
214 the 3'-termini of substrate labeled with a biotin that can bind to the fixed streptavidin,
215 and the substrate contained a DNA activator sequence for Cas12a at 5' termini. The
216 substrate-enzyme duplex remains stable due to its higher melting temperature, while a
217 low melting temperature between the DNA activator and the DNAzyme, ensured the

detachment of the DNA activator at room temperature (Xiong *et al.* 2020b). In the presence of target CEM or CIM of bacteria, DNAzyme cleaved a substrate and the DNA activator sequence was released, then Cas12a became activated and cut the fluorescent DNA reporters.

Aptamers, single-strand oligonucleotides, are able to be used as conformational switching motifs to construct ligand-responsive strategies in combination with CRISPR/Cas. Xie and colleagues reported a CRISPR/Cas12a-based bacteria detection platform based on a magnetic separation process (Li *et al.* 2020). The aptamer (Ab-Apt) of *Acinetobacter baumannii* (Ab) was immobilized on the surface of magnetic beads (MBs) via biotin-streptavidin interaction. A Cas12a activator-contained strand (c-Ab-Apt) was introduced to partially hybridize to the aptamer. The c-Ab-Apt would be released from the MBs in the presence of Abs, thus triggering the activation of Cas12a to output fluorescent signal. The LOD was estimated to be ~3 CFU/mL, which was close to the single-cell level. The magnetic separation, however, makes the experimental procedure complicated. The integration of two-dimensional materials with CRISPR/Cas systems could eliminate the magnetic separation process. Zhang *et al.* developed a rapid assay through coupling MXene with Cas12a for bacteria detection (Fig. 3D) (Sheng *et al.* 2021). The ASI/CSI probe consists of the target aptamer strand I (ASI) and corresponding complementary strand I (CSI), in which ASI contained the activating sequence for Cas12a to cut the ssDNA reporter. This turn-off strategy with ultralow background can quantify bacteria with detection limit of 23 CFU/mL.

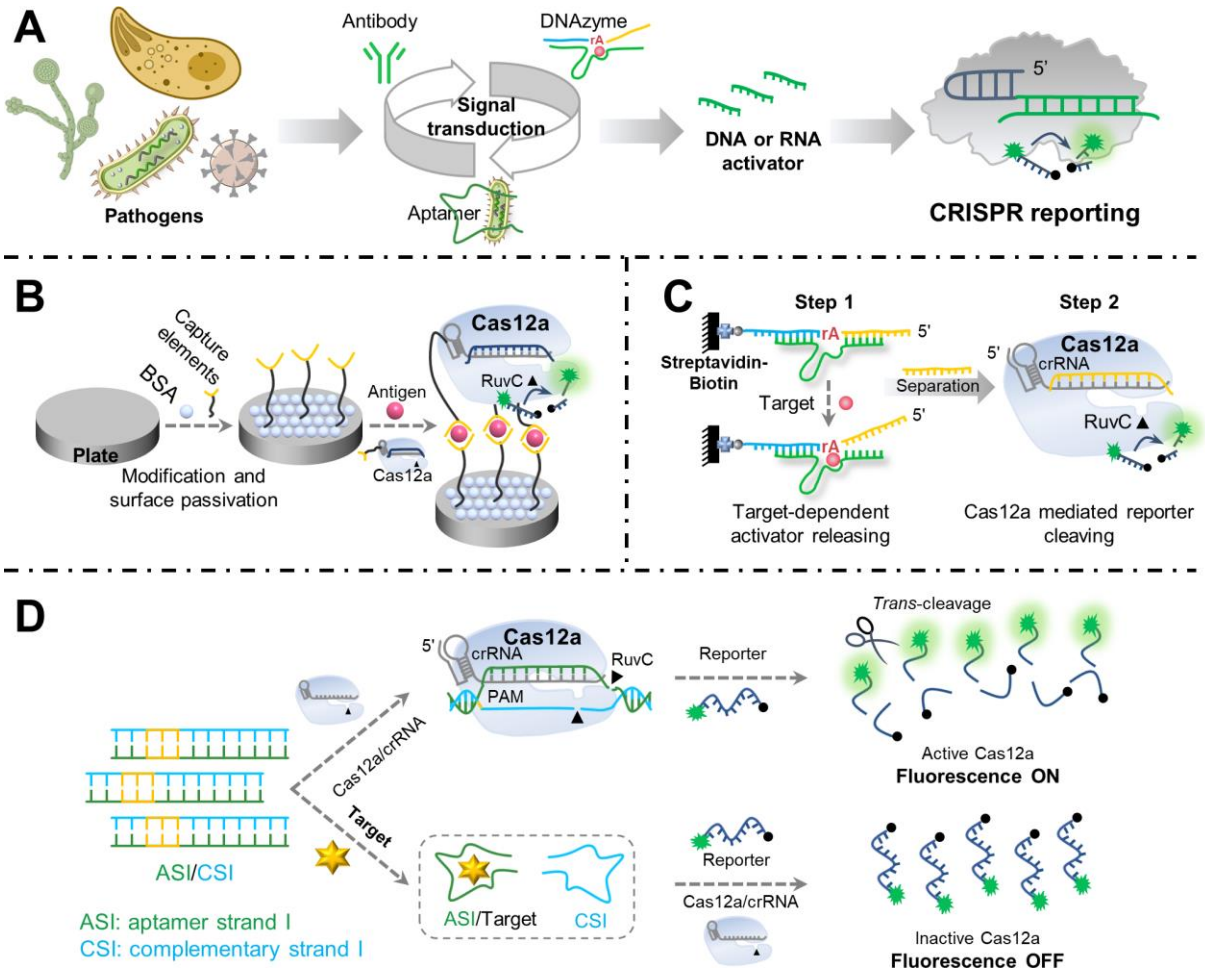


Fig. 3. CRISPR/Cas-based biosensors for analysis of pathogenic non-nucleic acid biomarkers. (**A**) Non-nucleic acid biomarker from pathogens needed to be recognised and converted into nucleic acid switch signals that can activate the subsequent CRISPR reporting process. (**B**) Antibody as the signal transduction element (Li *et al.* 2021c). Aptamer-linked Cas12a-based immunoassay for detection of non-nucleic acid analytes. (**C**) DNAzyme as the signal transduction element (Xiong *et al.* 2020b). DNAzyme regulates Cas12a sensors for pathogen detection. (**D**) Aptamer as the signal transduction element (Li *et al.* 2020). Combination of aptamer and Cas12a based versatile platform for sensitive bacteria quantification.

3. Strategies for POCT diagnosis

The direct capture of nucleic acids using Cas endonucleases without complicated purification and denaturation steps makes CRISPR tools compatible with POCT diagnosis, as they can rapidly provide easy-to-interpret results without sophisticated equipment. The fast and cost-effective readouts including fluorometry, colorimetry and lateral flow immunochromatography, were preferred for POCT diagnosis, achieving visual measurement with the assistance of LFA papers and mobile phones (Fig. 4) (Fozouni *et al.* 2021; Zhang *et al.* 2022a).

CRISPR-based fluorescent assays output signals via the collateral cleavage of ssDNA or ssRNA reporters carrying a pair of adjacent fluorophore and quencher, and the resulting fluorescence can be visualized by the naked eye under blue light (Fig. 4A, top). Inspired by that, Wang *et al.* proposed a Cas12a-based one-pot isothermal detection method (named Cas12aVDet) for on-site analysis of nucleic acids (Wang *et al.* 2019). Cas12VDet can accomplish the entire detection process within 30 min, and circumvent the uncapping contamination. Mobile phones are widespread, cost-effective, field-portable and easy to operate, thus are well suited for POCT diagnosis (Fig. 4A, bottom) (Kong *et al.* 2017; Vashist *et al.* 2014). Recently, an amplification-free Cas13a-based platform for on-site SARS-CoV-2 RNA detection without additional manipulation was developed and its signal could be read by a smartphone microscope (Fozouni *et al.* 2021). This assay, different from the other CRISPR-based techniques, did not require nucleic acid preamplification and multiple CRISPR/Cas cascade for signal amplification. Employment of multiple crRNAs targeting various loci can enhance Cas13a activity,

leading to an amplified signal output via multiple Cas13a proteins and their trans-cleavage activity. Combining with two crRNAs to target two loci markedly increased the detection sensitivity. Finally, it was proven that a mobile phone camera allowed 100 % accurate detection for 200 copies/μL and 50 % accuracy for 50 copies/μL over 30 min of measurement.

Colorimetric detection enables direct observation of the assay results with the naked eye. Metallic nanoparticles, such as gold nanoparticle (AuNPs) and silver nanoparticle (AgNPs), display distance- and size-dependent properties, thus are widely used in colorimetric biosensors (Mirkin *et al.* 1996). The CRISPR-mediated *trans*-cleavage of single-strand DNA/RNA linked to gold nanoparticles resulted in a vibrant colour in solution (Fig. 4B, top) (Zhou *et al.* 2023). Taking advantage of AuNPs to develop a programmable Cas12a-driven colorimetric coding strategy for highly rapid analysis of telomerase activity via naked-eye observation (Cheng *et al.* 2021). This assay involved three colorimetric codes that respond to positive, negative and false-negative results, respectively. Each code had two channels. Channel 1 contained Cas12a/crRNA1 in charge of distinguishing telomeric repeat DNA amplicons that indicated positive telomerase activity, and channel 2 contained Cas12a/crRNA2 responsible for monitoring internal control. Only lighting up of both two channels represented a positive result. Another strategy of colorimetric biosensors employs the enzymes (e.g., horseradish peroxidase, HRP) capable of catalyzing a chromogenic substrate (e.g., 3,3',5,5'-tetramethylbenzidine, TMB). Use of HRP-labeled oligonucleotides immobilized on the surface of magnetic beads serving as the reporter of Cas effectors can achieve target-

triggered colorimetric signal output (Fig. 4B, bottom) (Samanta *et al.* 2022). Benefitting from the high catalytical efficiency of HRP, the HRP-reporting CRISPR assay exhibited quick responsiveness (<1 h) and ~30-fold higher sensitivity (~10 fM) than that using conventional fluorophore/quencher reporter.

Colorimetric visualization can also be achieved by a chromogenic reaction. CRISPR processing produces a primer to initiate the subsequent amplification, leading to abundant G-quadruplex/hemin complexes (G4/hemin) that can convert the colorless ABTS-H_2O_2 to green color (Fig. 4C). A PAMmer-assisted Cas9 system specifically recognized and cleaved the target single-stranded nucleic acid, releasing a product fragment that can serve as the primer to activate subsequent exponential amplification reaction (EXPAR). G-rich EXPAR products were assembled with hemin to achieve a colorimetric detection (Wang *et al.* 2021b; Yin *et al.* 2021a). Moreover, Spoelstra *et al.* reported a CRISPR-assisted visual assay based on turbidity for nucleic acid detection, allowing to directly read the results by naked eyes in a tube (Spoelstra *et al.* 2021). In this assay, long oligonucleotides at high concentration caused the solution turbid and the turbidity can significantly decrease when the long oligonucleotides were largely cleaved by activated Cas proteins.

LFAs allow simple visualization using a reporter carrying fluorescein and biotin bound to anti-fluorescein isothiocyanate (FITC) antibodies coupled to gold nanoparticles in the sample pad of the strip (Fig. 4D). By coupling with LFAs, the Cas13a-based SHERLOCK platform allowed visual detection of ZIKV and DENV in patient samples

at a low concentration of 1 copy per microliter, and it enabled to detect Dengue virus from patient samples with no need for instrument in 2 h (Myhrvold *et al.* 2018).

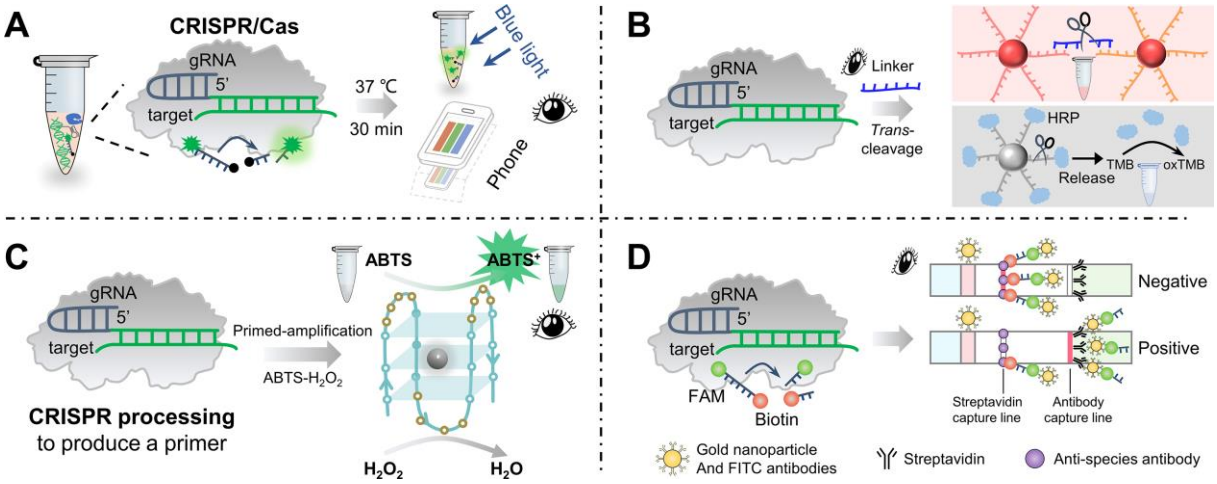


Fig. 4. Strategies for CRISPR-based POCT diagnosis. (**A**) CRISPR-based visual assay via fluorescence under a light excitation, the reporting signal can be observed by naked eyes or smartphones (Fozouni *et al.* 2021; Wang *et al.* 2019). (**B**) The CRISPR-mediated *tans*-cleavage towards the single-strand DNA/RNA linkers which behave as the junctions between gold nanoparticles or between magnetic beads and HRP, resulting in a colour change in solution (Cheng *et al.* 2021; Samanta *et al.* 2022). (**C**) CRISPR processing produces a primer to initiate the subsequent amplification, leading to produce abundant G4/hemin complexes that can catalyze the conversion of colorless ABTS-H_2O_2 into green (Wang *et al.* 2021b; Yin *et al.* 2021a). (**D**) LFAs contribute a simple visualization via taking advantage of reporters carrying fluorescein and biotin bind to anti-fluorescein isothiocyanate (FITC) antibodies coupled to gold nanoparticles in the sample pad of the strip (Myhrvold *et al.* 2018).

4. Pathogenic biosafety

A number of pathogens have been widely identified in clinical, food and environmental samples, serve as a critical biosafety issue that severely threatens human

health (Al Mughairy and Al-Lawati 2020). In order to effectively control their presence in clinic, food and environment, accurate and rapid analytical methods for monitoring these pathogens are in need of being developed and implemented. CRISPR-based analytical techniques have been widely exploited for pathogen diagnosis (Xia *et al.* 2021; Xiao *et al.* 2021). In this section, we highlighted current CRISPR-based biosensors for detecting bacteria, viruses, fungi and parasites, and their contributions to control pathogenic biosafety.

4.1. Bacteria

Information of pathogen's phenotypes and genotypes, such as the viability and drug-resistance, is important for estimating biosafety risks. In this section, we reviewed current CRIPSR-based biosensors for detecting bacteria as well as their key phenotypes and genotypes via detecting nucleic acid biomarkers with single-nucleotide resolution.

4.1.1. Bacteria cell

Predominantly, genetic testing has been considered the most reliable way for bacteria detection. Coupling with nucleic acid amplification, CRISPR-based assays allow a highly sensitive detection of bacteria and their genetic markers. Ascribing to the compatible condition of RPA amplification and Cas12a cleavage, the Cas12a-based RPA assay has been applied for the detection of bacteria with different sensing strategies, such as fluorescence (Wang *et al.* 2023), surface-enhanced Raman scattering (SERS) (Zhuang *et al.* 2022) and LFA (Liu *et al.* 2022a). For example, LFA-coupled assays for on-site detection of *Salmonella typhimurium* (*S. typhimurium*) (Zhuang *et al.* 2022) and

Staphylococcus aureus (*S. aureus*) (Liu *et al.* 2022a), and an one-pot fluorescent assay for POC testing of *Enterocytozoon hepatopenaei* infection in shrimp (Wang *et al.* 2023). The ratiometric biosensing can alleviate the cause of false-positive or negative results by measuring dual signals. Ma *et al.* adopted silver nanoclusters (AgNCs) as outputs to construct a Cas12a-based ratiometric fluorescent assay for detection of *S. typhimurium* (Fig. 5A) (Ma *et al.* 2023). A converter ssDNA was designed to complement the AgNCs probe, in which capable of converting green fluorescence emissive AgNCs to red emission. Degradation of converter ssDNA terminate the green-to-red fluorescent conversion, enabling ratiometric detection. This assay can detect *S. typhimurium* as low as 1 CFU/mL. Replacing Cas13a with Cas12a can evade the redundant transcription because of the DNA-targeted activation of Cas12a. Wei *et al.* coupled Cas12a with Ag^{+}-3,3',5,5'-tetramethylbenzidine (TMB) chromogen to construct a convenient colorimetric reaction for visual detection of *S. aureus* (Fig. 5B) (Wei *et al.* 2022a), posing the potential for POC application. The Ag^{+}-TMB which was pre-coupled to polycytosine-loaded magnetic beads served as the substrate of *trans*-cleavage by Cas12a.

Besides, using aptamers to directly recognize target bacteria followed by converting to nucleic acid signals allows to detect whole bacteria cells. An APC-Cas (allosteric probe-initiated catalysis and CRISPR-Cas13a) assay was established by employing Cas13a combined with a processed aptamer to detect *Salmonella enteritidis* cells in drinking water and milk (Shen *et al.* 2020). The allosteric probe contained three domains: aptamer domain, primer recognition domain and T7 promoter domain. In the presence of bacteria cells, the allosteric probe would unfold to unveil the primer binding site

domain and yielded dsDNA under the participation of DNA polymerase, followed by the release of target bacteria to bind the succeeding allosteric probe. Then, transcription amplification was conducted through the match between T7 promoter domain and T7 RNA polymerase, generating numerous ssRNAs which could activate CRISPR/Cas13a to cleave ssRNA reporters. The triple amplification of multiple-turnover recognition, transcription and Cas13a cleavage endows the APC-Cas system with single-molecular sensitivity.

4.1.2. Viable bacteria

Conventional culturing methods for viable pathogenic bacteria identification may fail due to the unculturability of viable but non-culturable (VBNC) pathogens. In addition, DNA targeting techniques may also fail to indicate whether the pathogen is alive or not because of the long-term existence of genomic DNA (Sheng *et al.* 2019; Trieu and Lee 2019; Váradi *et al.* 2017). RNA in dead cells is rapidly degraded, thus the presence of cellular RNA can indicate live bacteria. The emergence of RNA-targeting Cas13a opens possibilities for sensing applications in viable bacteria. Zhang *et al.* recently reported a light-up RNA aptamer signaling-Cas13a-based assay for label-free detection of viable pathogenic bacteria (*Bacillus cereus*) by directly targeting pathogen RNAs, achieving nucleic acid amplification-free and one-pot detection within 20 min (Fig. 5C) (Zhang *et al.* 2021). The introduction of a light-up RNA aptamer, broccoli contributed to eliminate the need for chemically labeled RNA reporters, and allowed reverse transcription-free analysis of RNA targets. Furthermore, combining NASBA preamplification with Cas13a

detection allowed to improve the sensitivity down to 1 CFU and 1 % viable bacteria (Xue *et al.* 2022).

Acquirement of spatial information of microbes and intra/inter-cell is conducive to understand the organization of microbial communities and cellular behaviors and functions such as antimicrobial resistance. *In vivo* imaging of bacterial genotype can provide insight into the complex microbiota and pathogenic risk (Shi *et al.* 2020). A polyethyleneimine-encapsulated CRISPR/Cas12a-circular reporter nanoprobes (CasCLR) tool was designed for rapid and direct *in vivo* visualization of genomic information in living bacteria (a clinically isolated *E. coli*) (Fig. 5D) (Li *et al.* 2022c). To enhance the stability of nucleic acid reporter in living bacteria, a circularization strategy was adopted, and circular reporter could be cleaved by Cas12a. Circularization of reporter improved signal-to-noise ratio by 14.5-fold than that in linearity. CasCLR enabled direct analysis of pathogenic bacteria in uncultured gut microbe of mouse.

The specific binding to the active proteins on the surface of viable bacteria cell enables aptamer to indicate the living bacteria state. An aptamer-based Cas14a platform for amplification-free detection of viable *S. aureus* with no need for nucleic acid extraction was developed (Fig. 5E) (Wei *et al.* 2022b). The binding of specific aptamer to viable *S. aureus* would release the blocker DNA from the pre-hybridized aptamer-blocker complex. The released blocker DNA could trigger the *trans*-cleavage of Cas14a under the guidance of sgRNA, generating a positive fluorescent signal. It allowed a low LOD of 400 CFU/mL viable bacteria cells and 100% detection accuracy in complex matrix comparable to qPCR.

4.1.3. Drug-resistant bacteria

The widespread prevalence of antimicrobial-drug-resistant bacteria resulted from the overuse of antibiotics. A magnetic relaxation switching strategy (C-MRS) based on Cas12a was demonstrated for the detection of methicillin-resistant *S. aureus* (MRSA) in food samples (Wei *et al.* 2023). Benefitting from the high catalytic activity of alkaline phosphatase, the C-MRS assay achieved preamplification-free detection. And the sequence specificity of Cas12a endowed C-MRS with the capacity of discriminating single-nucleotide variations. Capturing the cell information of spatial pattern and heterogeneity is the key approach to investigate cellular behaviors and functions such as drug-resistance. Xia *et al.* utilized the collateral cleavage of Cas12a to achieve single-cell *in situ* analysis of drug-resistant *Salmonella* by targeting the non-repetitive gene, *oqxB* (Xia *et al.* 2023). It was found that simultaneously targeting multiple loci could enhance the positive imaging signal, contributing to robust imaging of drug-resistant strains isolated in the clinic and intestinal tract sections. In addition, the *in situ* analysis assay allowed for investigation of the resistance of *Salmonella* towards to salt stress, and revealing a higher survival advantage of drug-sensitive strains than that drug-resistance strains.

Besides the plasmid-mediated drug-resistant bacteria, detection of SNP-involved drug-resistant strain could be more challenge. Yang *et al.* constructed a Cas12a-signaling amplification refractory mutation system (ARMS)-PCR (termed cARMS) assay, achieving specific and sensitive detection of SNP-involved *Salmonella enterica* (*S. enterica*) (Fig. 5F) (Yang *et al.* 2022b). High specificity of SNP recognition relied on

ARMS module which used an allele-specific forward primer with an artificial mismatch at the 4st position of the 3'-end. The following PCR amplification and Cas12a signaling conferred the cARMS assay with a sensitivity down to ~0.5 % comparable to qPCR. Particularly, the cARMS assay was applied to investigate the resistance of the drug-resistant bacteria to salt stress, and found a higher adaptation of drug-resistant strain than drug-sensitive one.

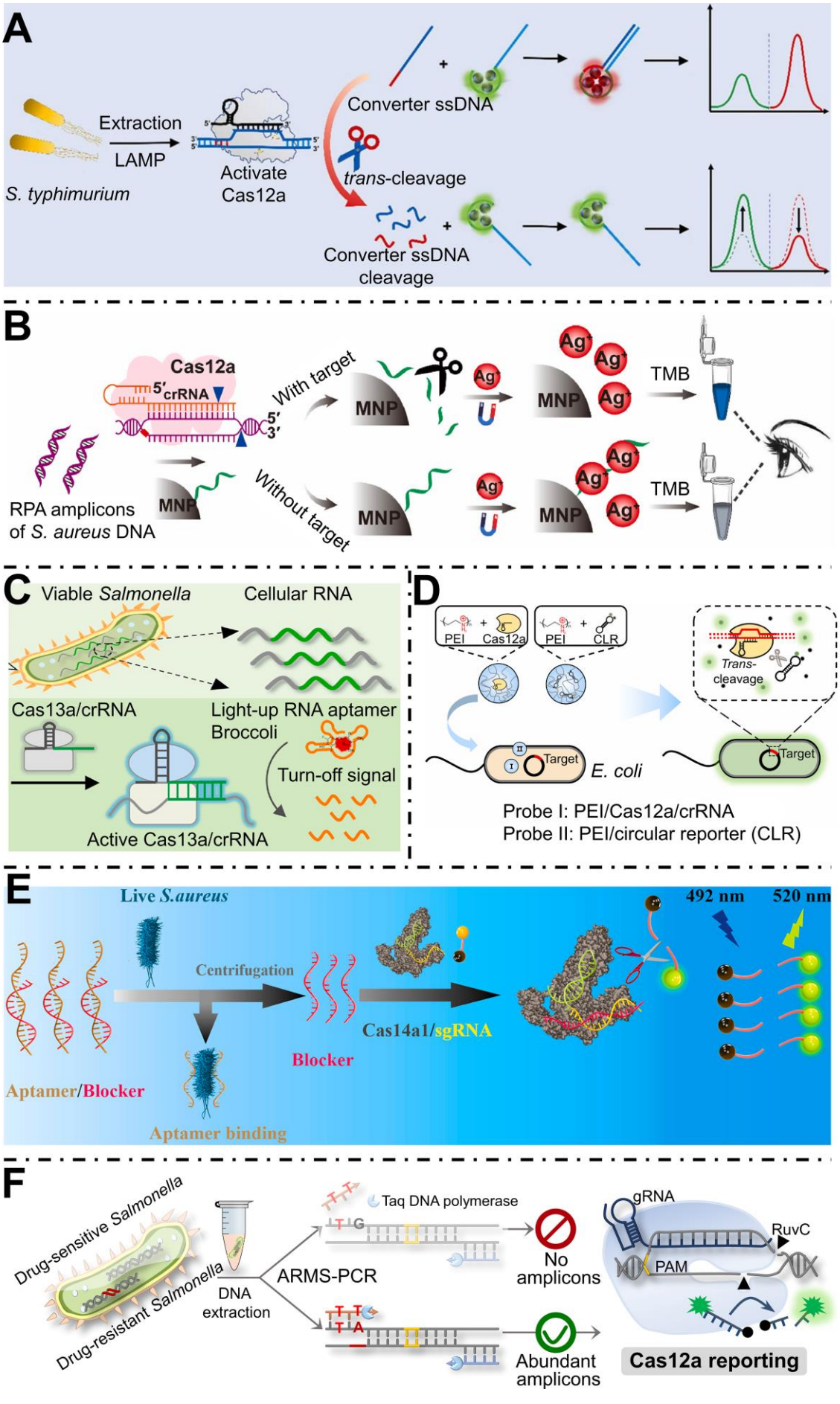

A
Extraction
LAMP
S. typhimurium
Activate Cas12a
Converter ssDNA
trans-cleavage
Converter ssDNA cleavage
B
Cas12a
5′ crRNA
5′ 3′
With target
Without target
MNP
Ag+
TMB
RPA amplicons of S. aureus DNA
C
Viable Salmonella
Cellular RNA
Cas13a/crRNA
Light-up RNA aptamer Broccoli
Turn-off signal
Active Cas13a/crRNA
D
PEI
Cas12a
CLR
Trans-cleavage
Target
E. coli
Probe I: PEI/Cas12a/crRNA
Probe II: PEI/circular reporter (CLR)
E
Live S.aureus
Centrifugation
Cas14a1/sgRNA
Blocker
Aptamer/Blocker
Aptamer binding
492 nm
520 nm
F
Drug-sensitive Salmonella
Drug-resistant Salmonella
DNA extraction
ARMS-PCR
Taq DNA polymerase
No amplicons
Abundant amplicons
gRNA
RuvC
PAM
Cas12a reporting

Fig. 5. CRISPR-based biosensors for detecting pathogenic bacteria. (A) AgCNs-based Cas12a assay for ratiometric detection of *S. typhimurium* (Ma *et al.* 2023). The dual green-to-red fluorescent outputs alleviated the cause of false-positive or negative results. (B) Colorimetric Cas12a assay using Ag^{+}-TMB reporter for detection of *S. aureus* (Wei *et al.* 2022a). The target-triggered color change of Ag^{+}-TMB reporter posed the potential for POC application. (C) Label-free Cas13a assay using light-up RNA aptamer broccoli as the reporter for direct detection of viable *Bacillus cereus* (Zhang *et al.* 2021). Use of RNA-targeting Cas13a evaded the application of RT. (D) Visualization of genomic information in living bacteria *E. coli* by Cas12a (Li *et al.* 2022c). Circularization of reporter improved the resistance to endonuclease in living cell. (E) An aptamer-based Cas14a platform for amplification-free detection of viable *S. aureus* (Wei *et al.* 2022b). Use of aptamer binding the active proteins on the surface of viable bacteria cell can indicate the living cellular state. (F) Cas12a signaling ARMS-PCR for the detection of SNP-involved drug-resistant *S. enterica* (Yang *et al.* 2022b). Dual recognition and amplification via ARMS-PCR and Cas12a binding enabled this assay high specificity.

4.2. Viruses

Pathogenic viruses can be grouped into two categories by the genetic material, DNA and RNA. DNA viruses include the grapevine red-blotch virus (GRBV) (Li *et al.* 2019c), white spot syndrome virus (WSSV) (Chaijarasphong *et al.* 2019) and African swine fever virus (ASFV) (He *et al.* 2020) that have been reported to be detected by CRISPR tools. Additionally, in the RNA viruses, norovirus is a foodborne virus that can causes over 90% of nonbacterial gastrointestinal diseases (Patel *et al.* 2009), particularly, the prevalent SARS-CoV-2 has been identified for person-to-person and material-to-person

transmission (Carducci *et al.* 2021; Yekta *et al.* 2021; Zhou and Shi 2021). Thus, developing techniques for detection of SARS-CoV-2 liked viruses is essential for identifying not only infected people but also contaminated foods and their surrounding environments.

4.2.1. DNA viruses

Considering the widespread infection of aquatic crustaceans with WSSV, Chaijarasphong *et al.* developed a Cas12a-based method for viral diagnosis by integrating nucleic acid amplification and Cas12a reporting. (Chaijarasphong *et al.* 2019). The described assay yielded a detection limit down to 200 copies WSSV per reaction and a sample-to-answer time of 1 h. Integrated visual reporting systems, such as color transition mediated by AuNPs, allows the Cas12a assays to be naked-eye detectable and field depolyable. The assay could achieve a detection limit of 40 pM, which was sufficient for the detection of GRBV infections in grapevine samples (Fig. 6A) (Li *et al.* 2019c). Nucleic acid preamplification may generate false-positive readouts resulted from nonspecific amplification and aerosol cross contamination, which severely impedes POCT application. Detection signal accumulation without nucleic acid preamplification can be achieved through the use of multiple crRNAs that target different loci. The high mortality (can reach 100 %) and outbreak rates of hemorrhagic African swine fever in both wild boars and domestic pigs make it urgent to exploit diagnostic technologies for ASFV control (Bai *et al.* 2019). Multiplex Cas12A/crRNA system enabled preamplification-free detection and showed ~64-fold improved

sensitivity (~1 pM) than the conventional single one for detecting ASFV (Fig. 6B) (Zeng *et al.* 2022). In combination with colorimetric module such as using urease catalysis, Cas12a detection can achieve a multiplexing and visual POCT diagnosis of ASFV with pM-sensitivity without nucleic acid preamplification (Fig. 6C) (Ki *et al.* 2022).

4.2.2. RNA viruses

Norovirus is a serious RNA virus that frequently occurs in public indoor spaces around the world mainly including schools and hospitals, through the consumption of Norovirus-contaminated food (Ahmed *et al.* 2014). Generally, RNA target first needs to be performed RT to obtain a corresponding DNA sequence. Qian *et al.* combined RT-RPA with Cas12a and LFA to develop a portable and reliable platform that enabled rapid (within 40 min) and sensitive (965 copies/mL) detection of human norovirus (Qian *et al.* 2021).

The global pandemic outbreak of 2019 novel coronavirus disease (COVID-19) makes an urgent demand for developing rapid, accessible and precise techniques for detection of the causative RNA virus, SARS-CoV-2 to reduce the risk of disease transmission. Dual-gene simultaneous detection is expected to improve accuracy and reduce false-negative results caused by partial gene digestion and amplification errors (Chen *et al.* 2022; Kim *et al.* 2020; Trémeaux *et al.* 2020). Such strategy has been developed for SARS-CoV-2 detection, where dual-Cas12a-based assay with the aid of an automated centrifugal microfluidics allowed on-site observation by the naked eye (Fig. 6D) (Chen *et al.* 2022). This "sample-to-answer" microfluidic platform can

automatically detect clinical SARS-CoV-2 samples with 100 % accuracy comparable to RT-PCR.

CRISPR-based detection technologies for viral variants of concern (VOCs), particularly the SARS-CoV-2 VOCs, have been sufficiently developed during the COVID-19 occurrence. Sui group introduced mismatches into crRNAs to improve allele discrimination, achieving the detection of SARS-CoV-2 *Spike* T478K, D614G, P681R, and P681H mutants in clinical samples with 70-78 % sensitivity and 100 % specificity (Fig. 6E) (Zhang *et al.* 2022b). And they further integrated an automatic digital microfluid into CRISPR-LAMP platform to realize labor-free and contamination-free detection. To meet SARS-CoV-2 diagnostics that are easy to use outside of centralized clinical laboratories, Myhrvold and colleagues reported one-pot SHERLOCK assay that allowed for RNA-extraction-free and isothermal POCT of SARS-CoV-2 and its VOCs, named SHINEv.2 (streamlined highlighting of infections to navigate epidemics, version 2). SHINEv.2 leveraged lyophilised reagents and rapid sample lysis at ambient temperature to eliminate the multiple heating steps and cold temperature storage requirement that SHINEv.1 suffered from (Fig. 6F) (Arizti-Sanz *et al.* 2022; Arizti-Sanz *et al.* 2020). SHINEv.2 enabled visual SARS-CoV-2 detection with 100% specificity and 90.5% sensitivity, and discrimination of SARS-CoV-2 VOCs including Alpha, Beta, Gamma, Delta and Omicron using LFA technology at body heat. Smartphones, integrating imageability, data analyzability, interactivity and potability can facilitate POCT. Ma *et al.* developed a AuNPs-based Cas12 colorimetric biosensing for POC diagnosis of SARS-CoV-2 using smartphone readout (Ma *et al.*

2022). It can detect 1 copy/μL pseudoviruses within 90 min, and provided 100% agreement with the qPCR test.

Compared with the gene testing, viral antigen detection using specific aptamer can reflect the viral infectivity and reduce contamination risk from enzymatic nucleic acid amplification. Zhang group reported a label-free and amplification-free electrochemical sensor employing aptamer to specifically recognize SARS-CoV-2 particles and Cas12a system to perform signal amplification (Fig. 6G) (Liu *et al.* 2022b). The competitive binding of specific aptamer to the nucleocapsid protein of SARS-CoV-2 released the activator of Cas12a, resulting significant signal change in impedance. This assay could complete the detection in 40 min without complex pretreatment process, and allowed a LOD of 0.077 ng nucleocapsid proteins per milliliter.

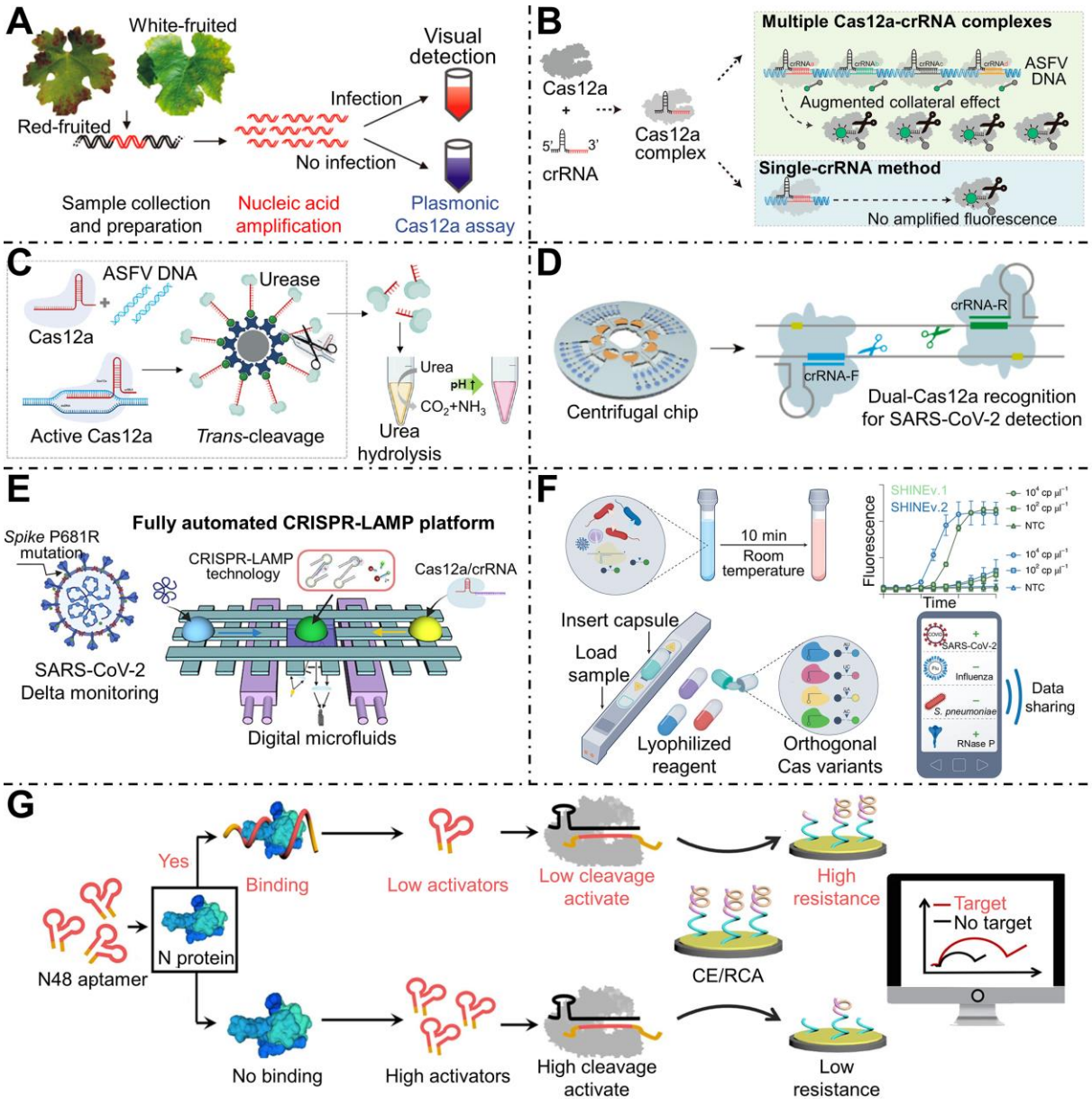


Fig. 6. CRISPR-based biosensors for detecting pathogenic virus. (A) AuNPs-based Cas12a assay for detection GRBV (Li *et al.* 2019c). Using DNA functionalized gold nanoparticles as the reporter of Cas12a cleavage enabled visual POCT diagnosis. (B) Multiple Cas12a/crRNA recognition for preamplification-free detection of ASFV (Zeng *et al.* 2022). Multiple Cas12a/crRNA recognition showed ~64-fold improved sensitivity than that using single crRNA for detecting ASFV. (C) Urease-catalyzed hydrolytic Cas12a assay for detection of ASFV (Ki *et al.* 2022). Using urease-catalyzed urea hydrolysis as reporter of Cas12a cleavage enabled visual POCT diagnosis. (D) Dual-Cas12a

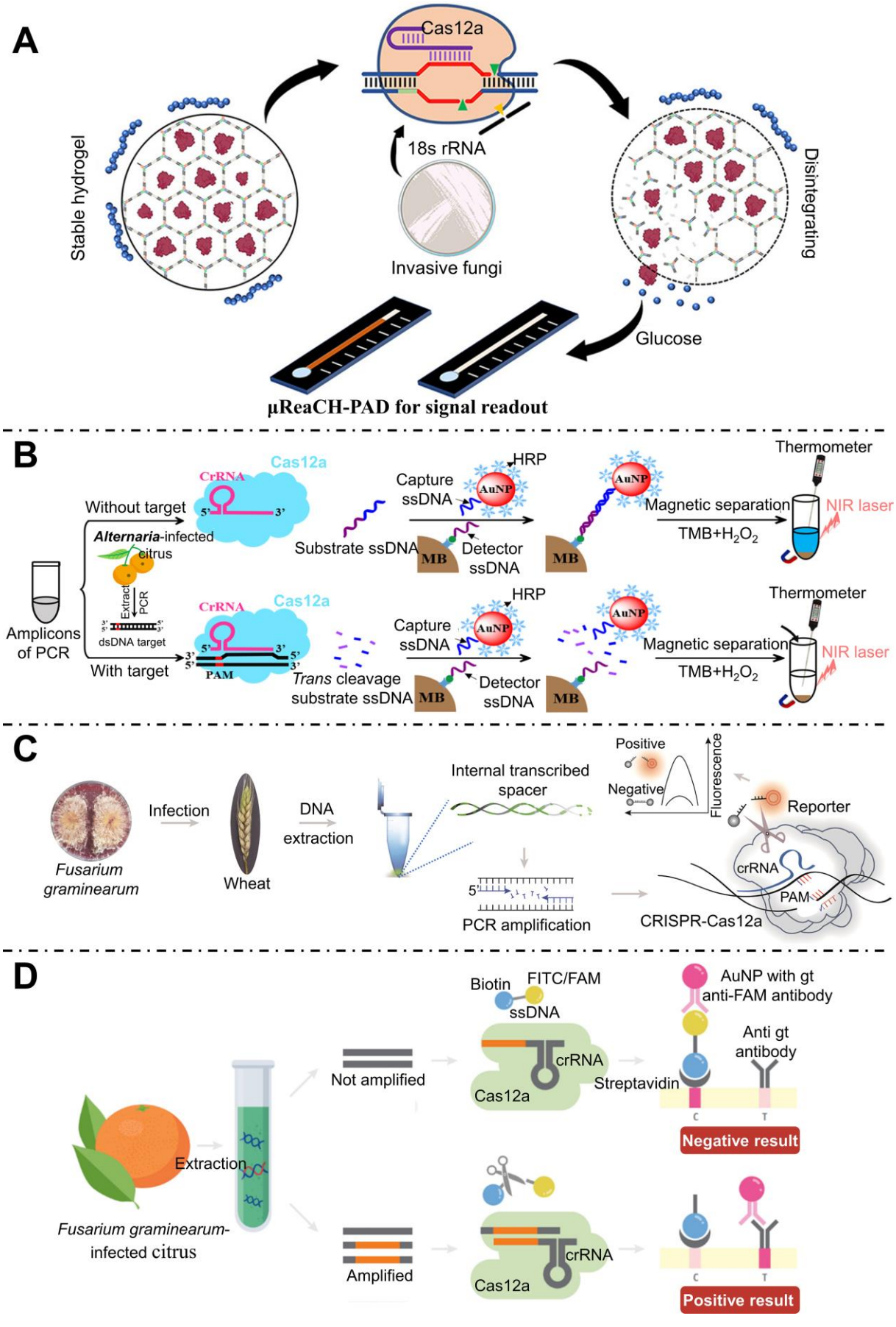
recognition for highly specific and sensitive detection of SARS-CoV-2 (Chen *et al.* 2022). Design of "sample-to-answer" microfluidic enabled automatically POC sensing. (E) CRISPR-LAMP platform with artificial mismatches for detection of SARS-CoV-2 VOCs (Zhang *et al.* 2022b). Use of digital microfluids enabled fully automated POCT diagnosis. (F) One-pot SHERLOCK assay (SHINE) for RNA-extraction-free and isothermal POCT diagnosis of SARS-CoV-2 and its VOCs (Arizti-Sanz *et al.* 2022). SHINEv.2 integrated lyophilised reagents and rapid sample lysis at ambient temperature, rendering it more feasible for POCT diagnosis than SHINEv.1. (G) Aptamer-based Cas12a assay for label-free detection of SARS-CoV-2 particles (Liu *et al.* 2022b). Use of aptamer enabled and RNA-extraction-free sensing of SRAS-CoV-2.

4.3. Fungi

Invasive fungal diseases have become a significant problem, not only causing severe loss of crop yields, but directly affecting human health. The commonly used clinical diagnostic culture-based method shows high accuracy but suffers from complicated operation and time-consumption, hampering the in-field testing (Safavieh *et al.* 2017). Another widely used method is morphology scanning, however, it cannot distinguish symptoms caused by various pathogens (Singha *et al.* 2016). Xu and colleagues developed a hydrogel paper-based analytical device using microfluidic ruler-readout and Cas12a-responsing for POCT diagnosis of invasive fungi (Fig. 7A) (Huang *et al.* 2021). Cas12a/crRNA was responsible for specifically targeting the genus-conserved 18s rRNA, subsequently using enzyme-caged DNA hydrogel for signal amplification and transduction, and microfluidic chips for visual quantification by naked eye. This assay can visually identify representative human fungal pathogens, *Candida* and *Aspergillus*,

as low as 10 CFU/mL. In addition, Liu *et al.* developed a Cas12a-based photothermal assay for POCT diagnosis. The assay involved only a thermometer, eliminating the need for cumbersome and expensive instruments traditionally used in morphological analysis and gene-sequencing. In the photothermal detection of *Alternaria* species using HRP-TMB reporter, the causative agents for citrus brown spot, this platform can detect target fungal genes as low as 1.5 pM in citrus, tomato, and apple samples (Fig. 7B) (Liu *et al.* 2022c).

Control of pathogenic fungal diseases in cereal crops requires reliable early diagnostic tools. As the primary causative agent of Fusarium head blight which is widespread in wheat-growing region, *Fusarium graminearum* (*F. graminearum*) may lead to severe yield reduction and generation of varieties of mycotoxins, contaminating food (Duan *et al.* 2014; Zhou *et al.* 2019). Classical PCR-coupled Cas12a-based nucleic acid assay was competent for the early diagnosis of scab of wheat caused by *F. graminearum* (Fig. 7C) (Mu *et al.* 2022). The assay allowed for highly specific discrimination of *F. graminearum* species and sensitive detection as low as 1 fg/μL total DNA, and it particularly can diagnose a 4-day infection of *F. graminearum*. Furthermore, fungal infection is a considerable problem in citrus, causing a significant drop in output quality. Shin *et al.* used a RPA-based Cas12a assay aided by LFA to achieve POC diagnosis of citrus scab (Fig. 7D) (Shin *et al.* 2021). The whole detection could be carried out within 1 h, and the LFA readout showed a high sensitivity down to 1 fg which allowed for robust detection even using crude DNA extracted from infected citrus tissue.


A
Cas12a
18s rRNA
Invasive fungi
Stable hydrogel
Disintegrating
Glucose
μReaCH-PAD for signal readout
B
Cas12a
CrRNA
Without target
Alternaria-infected citrus
Extract
PCR
dsDNA target
With target
PAM
Amplicons of PCR
Capture ssDNA
HRP
AuNP
Substrate ssDNA
MB
Detector ssDNA
Magnetic separation
TMB+H2O2
Thermometer
NIR laser
Trans cleavage substrate ssDNA
C
Infection
Fusarium graminearum
Wheat
DNA extraction
Internal transcribed spacer
PCR amplification
Positive
Negative
Fluorescence
Reporter
crRNA
PAM
CRISPR-Cas12a
D
Biotin
FITC/FAM
ssDNA
AuNP with gt anti-FAM antibody
Anti gt antibody
Streptavidin
Not amplified
Extraction
Fusarium graminearum-infected citrus
Amplified
Negative result
Positive result

593

Fig. 7. CRISPR-based biosensors for detecting pathogenic fungi. (A) Hydrogel paper-based Cas12a assay for detection of invasive fungi (*Candida* and *Aspergillus*) (Huang *et al.* 2021). Use of microfluidic ruler-readout enabled visual POC sensing by naked eyes. (B) Photothermal Cas12a assay for detection of *Alternaria* species in fruit (Liu *et al.* 2022c). Use of HRP-TMB reporter and thermometer enabled photothermal POCT diagnosis. (C) PCR-coupled Cas12a-based nucleic acid assay for detection of *F. graminearum* infection in wheat (Mu *et al.* 2022). This assay can diagnose a 4-day *F. graminearum* infection. (D) Cas12a-based RPA-assay for detection of *F. graminearum* infection in citrus (Shin *et al.* 2021). Use of LFA enabled POC diagnosis of citrus scab.

4.4. Parasites

Parasitic helminths are the causative agents of devastating chronic diseases, posing a major threat to public health and economic development in tropical and subtropical low-income communities in developing countries. Developing advanced parasites-diagnostic platforms compatible for use in resource-constrained settings is conducive to achieve eradication of parasitic infections. It is reported that SHERLOCK platform enabled sensitive detection and differentiation of the malaria-causative *Plasmodium* species including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* (Fig. 8A) (Lee *et al.* 2020). The platform particularly presented a 10-min rapid extraction protocol for parasites, assisting to achieve one-pot and isothermal detection. And the detection limit (0.36-2.4 parasites per microliter blood) for plasmodium species met the requirement (below two parasites per microliter blood) that was suggested by the WHO. Except for malaria infection, *Toxoplasma gondii* (*T. gondii*)-causing toxoplasmosis is an important zoonotic disease. *T. gondii* is an

opportunistic pathogen capable of infecting nearly 1/3 of human populations around the world (Zhou *et al.* 2011). Wang group utilized the RPA-Cas12a-based nucleic acid assay to detect *T. gondii* (Fig. 8B) (Lei *et al.* 2022). They designed a glass microfiber filter device for sample enrichment and lysis extraction, enabling nucleic acid extraction of low-concentration of *T. gondii*. Sample enrichment and signal amplification simultaneously contributed to a high sensitivity of the assay that yielded a LOD of 3.3 copies/μL. Employing fluorometer and LFA strip can realize the visual POC detection, benefiting the assay to be used in remote areas. Leishmaniasis is one of the most neglected tropical diseases around the world. Gao *et al.* recently developed a G-quadruplex-substrated Cas12a assay achieved label-free detection of *Leishmania donovani* with single-cell sensitivity (Gao *et al.* 2022). This assay can diagnose *Leishmania* infection in mice nearly 2 weeks earlier than the conventional parasitological analysis. dCas9 coupling with RCA can realize sensitive POCT diagnosis, Dekker et al used this strategy to isothermally detect the genus *Leishmania* with a sensitivity of 1 zeptomole (Fig. 8C) (Bengtson *et al.* 2022). The G-quadruplexes produced by RCA had peroxidase activity capable of catalyzing the color change of $ABTS^{2-}$ in the presence of hemin. This colorimetric readout achieved a visual detection with the naked eye.

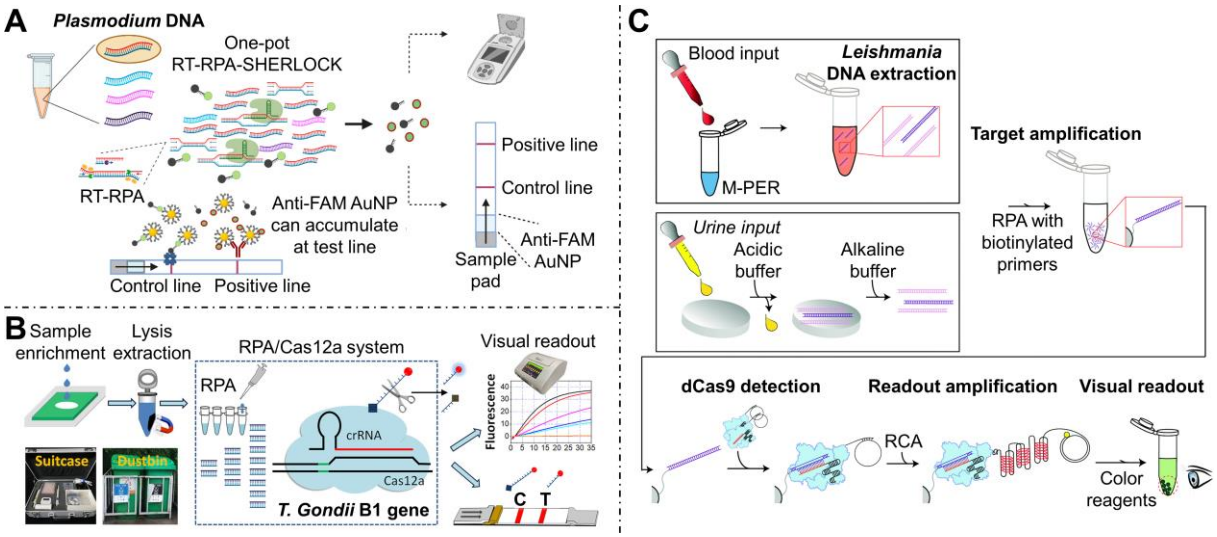


Fig. 8. CRISPR-based biosensors for detecting parasites. (A) One-pot RT-RPA-SHERLOCK assay for detection of the malaria-causative *Plasmodium* species (Lee *et al.* 2020). (B) Cas12a-based RPA assay for detecting *T. gondii* (Lei *et al.* 2022). Use of a glass microfiber filter device for sample enrichment and lysis extraction, enabling nucleic acid extraction of low-concentration of *T. gondii*. (C) dCas9 coupling with RCA for the detection of *Leishmania* (Bengtson *et al.* 2022). Use of G-quadruplexes DNAzyme enabled a visual detection.

641

Table 1. Overview of CRISPR-based biosensors for pathogen detection.

Cas protein	Analyte type	Amplification method	Auxiliary tool	Target	Assaying time	Sensitivity	Samples	Ref.
Cas9	Bacteria	RCA	MOF (UiO66)	*E. coli* O157:H7	120 min	10 CFU/mL	Spring water, skim milk and orange juice	(Sun *et al.* 2020b)
	Bacteria	-	Magnetic separation	MRSA	90 min	10 CFU/mL	-	(Guk *et al.* 2017)
	Fungus	RT-RPA	LFA strip	*Puccinia striiformis* f. sp. *tritici* and *Magnaporthe oryzae Triticum*	60 min	-	Infected wheat plants	(Sánchez *et al.* 2022)
	Parasite	RPA and RCA	G- quadruplex	*Leishmania*	-	1 zeptomole	Blood	(Bengtson *et al.* 2022)
Cas12a	Bacteria	RPA	Magnetic separation and aptamer	MRSA	95 min	8 CFU/mL	Clinical samples	(Wei *et al.* 2022a)
	Bacteria	RPA	Microfluidic paper	*S. typhimurium*	45 min	3-4 CFU/mL	Milk and meat	(Zhuang *et al.* 2022)
	Bacteria	-	Aptamer and biotin-streptavidin	*Acinetobacter baumannii*	30 min	3 CFU/mL	-	(Li *et al.* 2020)
	Bacteria	PCR	AuNPs	*Salmonella*	60 min	1 CFU/mL	Milk	(Ma *et al.* 2021a)

Bacteria	LAMP	G- Quadruplex	*S. enterica*	50 min	20 CFU	*In Vivo* colonization in chickens	(Xia *et al.* 2021)
Bacteria	RAA	Gold electrode	*L. monocytogenes*	10 min	26 CFU/mL	*Flammulina velutipes*	(Li *et al.* 2021b)
Bacteria	RCA	Antibody magnetic beads	*E. coli O157:H7*	100 min	10 CFU/mL	Milk	(Chen *et al.* 2021)
Bacteria	RPA	AuNPs, magnetic separation	*S. typhimurium*	60 min	1 CFU/mL	Milk	(Cai *et al.* 2021)
Bacteria	PCR/RAA	LFA strip	*S. aureus*	60 min	1 CFU/reaction	Milk	(Qian *et al.* 2022)
Bacteria	Isothermal amplification	Aptamer and Au electrode	*E. coli O157:H7*	120 min	19 CFU/mL	Milk	(Bu *et al.* 2021)
Bacteria	RPA	-	*Enterocytozoon hepatopenaei*	40 min	10 gene copies per reaction	Shrimp	(Wang *et al.* 2023)
Bacteria	HCR	All-fiber	*E. coli* O157:H7	50 min	17.4 CFU/mL	Tap water and secondary sedimentation tank effluent	(Song *et al.* 2023)
Bacteria	LAMP	Membrane filter	*E. coli O157:H7*	70 min	1.22 CFU/mL	Romaine Lettuce	(Lee and Oh 2022)
Bacteria	RAA	LFA strip	*S. aureus*	70 min	540 CFU/mL	Beef, pork, tomato, coriander and lettuce	(Zhou *et al.* 2022)
Bacteria	NEAA	-	*Salmonella*	20 min	80 CFU/mL	Egg	(Bai *et al.* 2022)
Bacteria	NASBA	-	*S. enterica*	150 min	1 CFU	Egg shell, beef, pork, duck, chicken, mutton and tap water	(Xue *et al.* 2022)

Bacteria	-	Magnetic separation	MRSA	100 min	16 CFU/mL and 0.01 % SNP	Eggs, milk and pork	(Wei *et al.* 2023)
Bacteria	LAMP	Silver nanoclusters	*S. typhimurium*	55 min	1 CFU/mL	Orange juice and eggs	(Ma *et al.* 2023)
Bacteria	PCR or RPA	-	Drug-resistant *Salmonella*	45 min	1 CFU/mL	Milk and skim milk powder	(Fu *et al.* 2022)
Bacteria	ARMS-PCR	-	*S. enterica*	60 min	~0.5 % SNP	-	(Yang *et al.* 2022b)
Virus	PCR	AuNPs	GRBV	30 min	40 pM	Grapevine	(Li *et al.* 2019c)
Virus	-	Multiple crRNAs	ASFV	90 min	1 pM	-	(Zeng *et al.* 2022)
Virus	-	Magnetic beads and urease	ASFV	60 min	50 pM	Porcine clinical tissue	(Ki *et al.* 2022)
Virus	RAA		African swine fever virus	60 min	10 aM	Pig blood	(Bai *et al.* 2019)
Virus	PCR or RPA	-	WSSV	60 min	200 copies per reaction	Shrimp	(Chaijaraspong *et al.* 2019)
Virus	RT-RPA	LFA strip	Norovirus	40 min	965 copies/mL	Clinical samples from patients with gastroenteritis	(Qian *et al.* 2021)
Virus	RT-RPA		SARS-CoV-2	45 min	10 copies	Clinical samples	(Meng *et al.* 2021)
Virus	RAA	Microfluidics	SARS-CoV-2	30 min	1 copy/μL	Throat swab	(Chen *et al.* 2022)
Virus	LAMP	Microfluidics	SARS-CoV-2	45-75 min	100 copies/μL	Nasopharyngeal swab	(Zhang *et al.* 2022b)

Virus	RT-PCR	AuNPs	SARS-CoV-2	90 min	1 copy/μL	Throat swab	(Ma *et al.* 2022)
Virus/viroids	RT-RPA	AuNPs	Multiple prevalent RNA viruses/viroids in apple	30 min	250 or 2500 viral copies per reaction	Crude apple leaf	(Jiao *et al.* 2021)
Fungus	RAA	Microfluidic chips	*Candida* and *Aspergillus*	90 min	4.90 and 4.13 CFU/mL	Ascites, wound discharge, urine, sputum, blood	(Huang *et al.* 2021)
Fungus	PCR	Magnetic beads, AuNPs and thermometer	*Alternaria*	110 min	1.5 pM	Citrus, apple and tomato	(Liu *et al.* 2022c)
Fungus	PCR	-	*F. graminearum*	120 min	1 fg/μL	Wheat	(Mu *et al.* 2022)
Fungus	RPA	LFA strip	*Elsinoë fawcettii*	150 min	1 fg	Citrus	(Shin *et al.* 2021)
Parasite	RT-RPA	LFA strip	*Plasmodium*	70 min	<2 parasites per microliter blood	Blood	(Lee *et al.* 2020)
Parasite	RPA	LFA strip	*T. gondii*	55 min	3.3 copies/μL	Heavily polluted landfill leachate	(Lei *et al.* 2022)
Parasite	RAA	-	*T. gondii*	70 min	1 fM	Soil	(Ma *et al.* 2021b)
Parasite	RPA	LFA strip	*Cryptosporidium parvum*	90 min	1 copy of GP60 gene and 10 oocysts/g	Human and cattle fecal	(Yu *et al.* 2021)
Parasite	RPA	G-quadruplex	*Leishmania donovani*	120 min	3.1 parasites	Infected mice spleen samples	(Gao *et al.* 2022)

Cas13a	Bacteria	Transcription	Aptamer	*Salmonella* Enteritidis	60 min	1 CFU	Milk and serum	(Shen *et al.* 2020)
	Bacteria	PCR and transcription	-	*S. typhimurium*	120 min	1 CFU/mL	Milk and juice	(Gao *et al.* 2021)
	Bacteria	RAA	Microfluidic chip	*Listeria*	60 min	12.1-72.1 aM	*Flammulina velutipes*	(Xiang *et al.* 2022)
	Bacteria	-	Light-up RNA aptamer	*Bacillus cereus*	20 min	10 CFU	Rice and milk	(Zhang *et al.* 2021)
	Virus	Transcription	Light-up RNA aptamer	SARS-CoV-2	180 min	82 copies	Food packages, seafood and throat swabs	(Wang *et al.* 2021c)
	Virus	RT-RPA and transcription	LFA strip	SARS-CoV-2	90 min	200 copies/μL	Nasopharyngeal swabs	(Arizti-Sanz *et al.* 2022)
	Virus	RT-PCR	Microfluidic chip	SARS-CoV-2	180 min	1000 copies/μL	Nasopharyngeal swabs	(Welch *et al.* 2022)
	Virus	-	-	SARS-CoV-2	35 min	84 aM	Saliva samples	(Yang *et al.* 2022c)
	Parasite	RPA and transcription	-	*Plasmodium*	210 min	2.5-18.8 parasites/μL	Blood	(Cunningham *et al.* 2021)
Cas14a	Bacteria	RT-asymmetric PCR	-	*Eberthella typhi* and *Streptococcus pyogenes*	60 min	10^4 CFU/mL	Milk	(Ge *et al.* 2021)
	Bacteria	RT-PCR	Magnetic bead	*E. coli*	60 min	1 CFU/mL	Clinical blood	(Song *et al.* 2021)

	Bacteria	-	Aptamer	*S. aureus*	210 min	400 CFU/mL	Coconut water and milk	(Wei *et al.* 2022b)

642

5. Concluding remarks and perspectives

Analytical tools for pathogenic biosafety have advanced rapidly. Among them, CRISPR-based biosensors, derived from synthetic biology tools, are of growing interest due to their specific recognition capability, high cleavage activity and easy programmability. As shown in Table 1, these assays have evolved into multitudes of analytical techniques for rapid, sensitive and on-site detection of different pathogens associated with biosafety. Particularly, the COVID pandemic accelerate the development of CRISPR-based biosensors for POCT application. However, several challenges remain in applying CRISPR-based biosensors for practical and commercialized use. First, in field diagnostics, the complex matrix derived from samples may inference the amplification and signal yield, so pretreatments such as extraction and purification are usually needed. Development of rapid and convenient extraction devices, such as microneedles (Paul *et al.* 2019) and microfiber filters (Lei *et al.* 2022), can facilitate the CRISPR-based biosensors for on-site detection. Second, the emerging pathogenic variants, particularly those associated with single-nucleotide mutation, highlight the demand for nucleic acid assays with single-nucleotide resolution. The design of chemical modification and artificial mismatches in gRNA (Rozners 2022; Yang *et al.* 2022a), as well as engineered Cas proteins (such as split Cas12a) (Nihongaki *et al.* 2019) hold the potential to improve the assaying specificity to discriminate mutations in pathogenic variants. Third, preamplification makes the detection process complicated, time-consuming, and possibly induces false-positive caused by contamination. Strategies such as the use of multiplex Cas effectors, cascade CRISPR

assays (Gootenberg *et al.* 2018; Sha *et al.* 2021; Shi *et al.* 2021) and droplet microfluidic platforms (Sun *et al.* 2020a; Tian *et al.* 2021) may enable nucleic acid preamplification-free detection with femtomolar sensitivity. Fourth, pathogenic variants involve multiple mutations and infections may be caused by different pathogens, thus highlighting the development of multiplexing assays. Different targeting Cas effectors and reporting approaches can be exploited to meet the multiplex detection, where the class 1 CRISPR system (Mohanraju *et al.* 2022) and protein reporter-based CRISPR system (e.g., Craspase) (Hu *et al.* 2022) could be candidates. In addition, it is necessary to seek and develop new signal transduction elements that can be combined with CRISPR system for non-targeted screening of pathogens, as risky pathogens are rapidly emerging. The development of enzyme engineering, nanotechnology, and microfluidic techniques would facilitate the CRISPR system to serve as critical tools for pathogenic biosafety monitoring.

CRediT author contribution statement

Hao Yang: Conceptualization, Writing – original draft, Writing – review & editing. **Rodrigo Ledesma-Amaro**: Writing – review & editing. **Hong Gao**: Writing – review & editing. **Yao Ren**: Conceptualization, Supervision, Writing – review & editing. **Ruijie Deng**: Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of competing interest

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

Acknowledgement

This work was financially supported by National Natural Science Foundation of China (No. 22074100), the Young Elite Scientist Sponsorship Program by CAST (no.YESS20200036), the Green Manufacturing Project of Ministry of Industry and Information Technology of China.

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