



Cite this: *Chem. Commun.*, 2025, 61, 1748

Surveillance of antimicrobial resistance using isothermal amplification: a review

Menghan Hou,^a Xinying Yang,^a Lin Gong^{ab} and Xiantao Shen^{id}*^a

The monitoring of antibiotic resistance genes (ARGs) is crucial for understanding the level of antimicrobial resistance and the associated health burden, which in turn is essential for the control and prevention of antimicrobial resistance (AMR). Isothermal amplification, an emerging molecular biology technology, has been widely used for drug resistance detection. Furthermore, its compatibility with a range of technologies enables high-specificity, high-throughput, and portable and integrated detection in drug resistance, particularly in resource-limited areas. However, to date, reviews involved in isothermal amplification all concentrate on its technological advancements and its application in nucleic acid point-of-care testing. Few reviews have been published that focus specifically on the application of isothermal amplification in the detection of drug resistance. This review summarizes the detection principles of different isothermal amplification techniques and discusses their strengths and weaknesses as well as the applicable scenarios for drug resistance detection. It also summarizes advances in the application, challenges and prospects of isothermal amplification technologies in conjunction with different methods such as base mismatch, CRISPR-Cas, lateral flow immunoassay, sensing and microfluidic technologies for improvement of specificity, throughput and integration for drug resistance detection. It is anticipated that this review will assist scientists in comprehending the evolution of isothermal amplification in the context of drug resistance detection and provide insights into the prospective applications of isothermal amplification for highly integrated and immediate on-site detection of drug resistance.

Received 15th October 2024,
Accepted 19th December 2024

DOI: 10.1039/d4cc05488a

rsc.li/chemcomm

1 Introduction

Antimicrobial resistance represents one of the most significant global public health threats of the 21st century, and has given rise to widespread concern.¹ It is estimated that approximately 700 000 deaths per year are attributable to AMR, with this figure projected to reach 10 million by 2050.² In order to address the global issue of AMR, scientists have put forth a “One Health” approach as a potential solution.³ One of the key components of this solution is the monitoring of ARGs, with the aim of gaining deeper insights into the level of AMR and the associated health burden.⁴ Moreover, given the high mutation rate,⁵ diversity/multi-drug resistance,⁶ and rapid spread of resistance genes,⁷ detection technologies of AMR must meet the requirements of high specificity, high sensitivity, and high throughput. Furthermore, in consideration of the inherently unstable

nature of samples carrying ARGs, which are susceptible to mutation during the processes of transport and storage,⁸ an optimal AMR assay should also have the potential for immediate detection in the field, with the objective of ensuring accuracy and comprehensive applicability.

At present, the most commonly used methods for detecting bacterial resistance are based on antimicrobial susceptibility testing and polymerase chain reaction (PCR) techniques,^{9–13} including quantitative PCR, digital PCR, reverse transcription PCR, and macro gene sequencing.⁷ However, the antimicrobial susceptibility testing for drug resistance analysis is a laborious and time-consuming process, which results in delays to the timeliness and usability of the results. In addition, the detection sensitivity is contingent upon the enriched nature of the culture,⁹ necessitating subsequent molecular confirmatory testing. Although molecular biology based on PCR and gene sequencing for drug resistance analyses have the potential to reduce detection times and facilitate high-throughput analysis, they frequently necessitate the use of sophisticated instrumentation and trained personnel,¹³ which can limit their applicability. It is evident that none of the aforementioned methods are capable of fulfilling the requisite demands for immediate on-site detection of ARGs.

Isothermal amplification, a recently developed molecular biology technique, has also been employed for the immediate

^a State Key Laboratory of Environment Health (Incubation), Key Laboratory of Environment and Health, Ministry of Education, Key Laboratory of Environment and Health (Wuhan), Ministry of Environmental Protection, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Hangkong Road #13, Wuhan, Hubei 430030, China.
E-mail: xtshenlab@hust.edu.cn

^b Department of Disinfection and Pest Control, Wuhan Center for Disease Control & Prevention, Wuhan, Hubei, 430000, China

Highlight

on-site detection of AMR,¹⁴ due to its mild reaction conditions, no requirement of equipment, and short reaction time. Currently, a variety of isothermal amplification techniques are applied for the monitoring of AMR, including loop mediated isothermal amplification (LAMP),¹⁵ recombinase polymerase amplification (RPA),¹⁶ and rolling circle amplification (RCA).¹⁷ Moreover, its high compatibility with a variety of technologies enables high-specificity,¹⁸ high-throughput,¹⁹ and portable and integrated detection of AMR,²⁰ thereby facilitating the advancement of drug resistance monitoring, particularly in resource-limited regions.

In the past few decades of development, many studies involving isothermal amplification techniques in the detection of AMR have been reported. However, to the best of our knowledge, reviews involved in isothermal amplification all concentrate on its technological advancements^{21–23} and its application in nucleic acid point-of-care testing.²⁴ There is still a lack of review on isothermal amplification technology in AMR monitoring. In this case, a review article to introduce the surveillance of ARGs using isothermal amplification is fortuitously timed.

Therefore, this review introduced three different isothermal amplification techniques and provided an analysis of the advantages and disadvantages of existing isothermal amplification techniques in the detection of AMR. Furthermore, the isothermal amplification in conjunction with alternative methods for enhancing the detection of drug resistance was also reviewed from three aspects: specificity, throughput and integration. And a special emphasis was given to the developmental potential of the use of microfluidics in conjunction with isothermal amplification technology in the development of immediate resistance detection. Finally, some potential challenges and application prospects of isothermal amplification in

drug resistance detection were also discussed. We believe that this review would assist in comprehending the utility of isothermal amplification in the context of drug resistance detection, while also offering insights into the prospective evolution of isothermal amplification technology for highly integrated, immediate on-site detection of drug resistance (Fig. 1).

2 Overview of isothermal amplification techniques

Isothermal amplification, which was developed from the PCR, allows the expansion of the copy number of a specific DNA or RNA fragment at a particular temperature.²⁵ This is achieved through the use of enzymes with varying activities and their respective specific primers. Similar to the PCR, isothermal amplification assays are commonly used in fluorescent dye and fluorescent probe methods.^{26,27} In comparison to the PCR, isothermal amplification technology does not necessitate the use of costly equipment for temperature control, resulting in its more straightforward operation, shorter detection time, and compatibility with a range of assays.^{28,29} Furthermore, isothermal amplification can be integrated with a portable detection device for the interpretation of results.³⁰ Therefore, it is more advantageous to develop on-site detection for AMR. The most commonly employed isothermal amplification techniques for the detection of drug resistance are LAMP, RPA and RCA. The following section will describe the principles and characteristics of these techniques in the context of AMR detection.

2.1 Principles of LAMP technology and its characteristics in AMR detection

LAMP is an isothermal amplification technique that was first reported in 2000.³¹ The reaction process can be divided into three phases: a cyclic template synthesis phase, a cyclic amplification phase, and an elongation and recirculation phase. The fundamental principle is to utilize Bst DNA polymerase and two distinct pairs of primers (comprising one pair of internal primers and one pair of external primers) for the identification of six specific regions of the target gene (F1-F1c, F2-F2c, F3-F3c, B1-B1c, B2-B2c, B3-B3c). Subsequently, strand displacement reaction occurs and continues, generating large amounts of polycyclic cauliflower structural DNA of varying lengths in a relatively short period of time, thereby achieving the specific amplification of the target genes at a temperature of 65 °C.³² Furthermore, two loop primers were added to the LAMP system by Nagamine,³³ which could bind to stem-loop DNA and thus enhance the strand displacement efficiency, thereby reducing the duration of the LAMP reaction. For a better understanding of LAMP, the amplification processes are illustrated in Fig. 2.

In our opinion, the LAMP technique is distinguished by its high specificity and high amplification efficiency, which contribute to its successful application in the detection of AMR.^{15,34,35} LAMP also features visual detection, based on the combination of the by-product pyrophosphate with magnesium

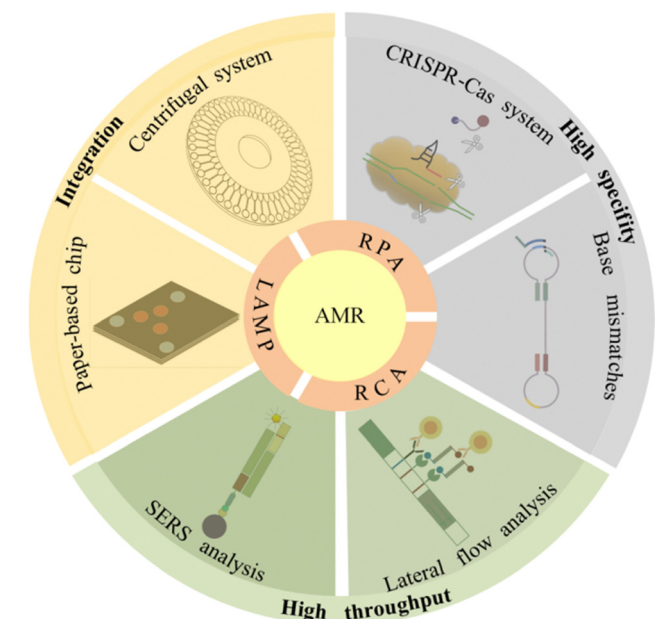


Fig. 1 Schematic representation of base mismatch, CRISPR-Cas, lateral flow immunoassay, sensor technology and microfluidic coupled with isothermal amplification techniques applied to antimicrobial resistance monitoring.

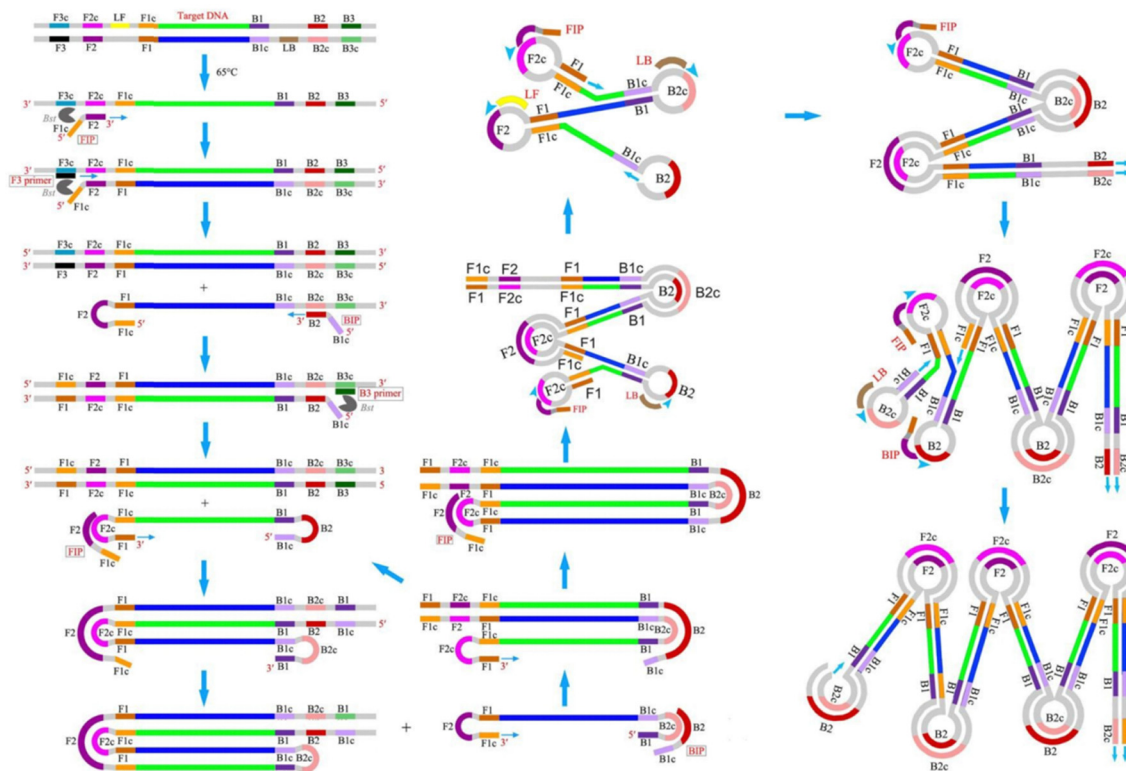


Fig. 2 Loop-mediated isothermal amplification scheme. Reproduced with permission.³² Copyright 2016, Frontiers.

ions during the amplification phase, which results in the formation of a white precipitate.³⁶ Alternatively, during the same phase, the combination of hydrogen ions with added acid–base indicators also produces a colour change. These properties offer the potential for immediate detection of ARGs, which is particularly advantageous in resource-limited settings. Furthermore, the incorporation of fluorescent probes into the amplification system for fluorescence detection can enhance the specificity of the LAMP assay.³⁷

Nevertheless, the design of LAMP amplification primers is complex, and single-tube multiple amplification is challenging, which is not conducive to the high-throughput detection of ARGs. The length of LAMP-amplified fragments is typically 200–300 bp, and the process is susceptible to contamination, which can result in false-positive outcomes. These present a challenge in the application of LAMP for drug resistance detection.

2.2 Principles of RPA technology and its characteristics in AMR detection

The concept of RPA was first proposed by Piepenburg in 2006.³⁸ The fundamental mechanism underlying this process is as follows: firstly, the recombinant enzyme protein T4uvsX binds to the primer to form a nucleic acid–protein complex with the action of cofactors. Secondly, the complex undergoes hybridization with the target DNA, accompanied by a process of strand replacement. Subsequently, the replaced single strand is bound by single-stranded binding protein named gp32 to prevent rehybridization. Finally, the recombinant enzyme is dissociated

under the action of ATP, allowing Bsu DNA polymerase to bind to the primer and extend along the template, thereby achieving the isothermal amplification of the target gene.³⁹ A schematic diagram of an RPA system is illustrated in Fig. 3. The amplification reaction can be conducted at 37 °C, with a sensitivity of up to a single copy and an amplification product length within 500 bp.

In comparison to LAMP, the RPA technique is also frequently employed for the detection of drug resistance due to its lower reaction temperatures.^{18,19,40,41} Since its ambient amplification characteristics, it has greater compatibility and is more conducive to concatenation with other methods,^{42–44} thereby improving detection performance. The design of a primer for RPA is relatively straightforward, and multiplex amplification is less challenging than LAMP. These characteristics render it a promising candidate for further method development in the context of high-throughput, portable drug resistance detection. Nevertheless, the lengthy primer (30–35 nt) and probe (46–54 nt) sequences necessitated by the RPA reaction are ill-suited to the detection of shorter target sequences.¹⁸ Furthermore, the rigorous conditions of the RPA reaction, along with the costs and preservation of enzymes, have also impeded the advancement of RPA for rapid on-site drug resistance detection to a certain degree.

2.3 Principles of RCA technology and its characteristics in AMR detection

The RCA technique was initially proposed by Fire in 1995 and draws upon the microbial circle DNA replication process.⁴⁵ The

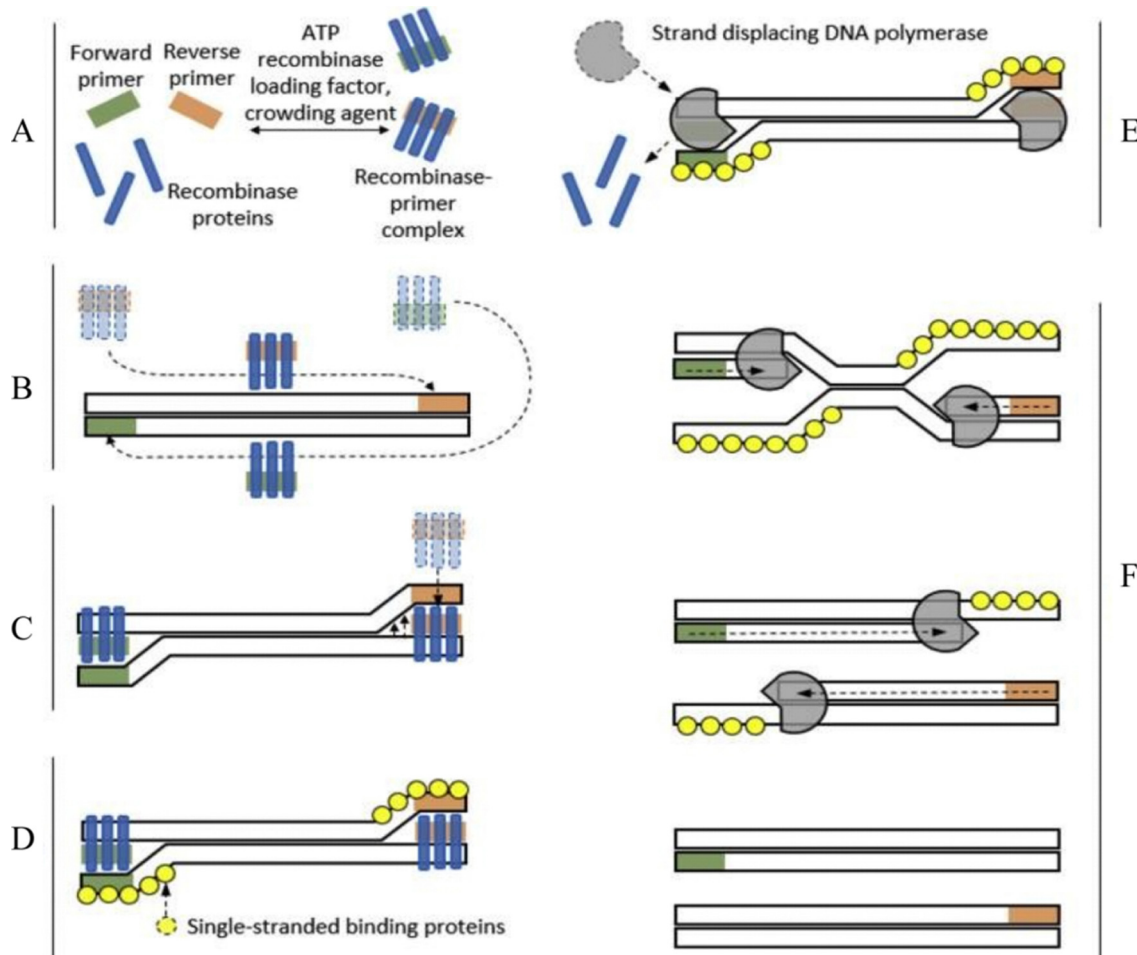


Fig. 3 RPA amplification scheme. Recombinase proteins form complexes with each primer (A), which scans DNA for homologous sequences (B). The primers are then inserted at the cognate site by the strand-displacement activity of the recombinase (C) and single stranded binding proteins stabilize the displaced DNA chain (D). The recombinase then disassembles leaving the 3'-end of the primers accessible to a strand displacing DNA polymerase (E), which elongates the primer (F). Exponential amplification is achieved by cyclic repetition of this process. Reproduced with permission.³⁹ Copyright 2018, Elsevier.

basic principle is to use circular DNA as a template, and convert dNTPs into single-stranded DNA catalysed by phi29 DNA polymerase through a short DNA primer (complementary to a portion of the circular template), which contains hundreds of repeats of the template's complementary fragments.⁴⁶ The fundamental principle of RCA is illustrated in Fig. 4. RCA can be classified into three categories: single-primer RCA, double-primer RCA, and multi-primer RCA.⁴⁷⁻⁴⁹ These distinctions are based on the number of primers involved. The formation of a perfectly matched target-probe complex during target gene cyclization effectively circumvents the non-specific amplification that may occur in traditional PCR.⁵⁰ This results in an extremely high degree of specificity, allowing for the accurate identification of gene mutations, including single nucleotide polymorphisms (SNPs).^{51,52} Additionally, this method possesses a distinctive advantage in the detection of drug-resistant genes.

In comparison to the other two amplification methods, the reaction mechanism of RCA is relatively straightforward,

necessitating only a single polymerase for amplification at room temperature. It exhibits high sensitivity, with the amplification efficiency of a single primer reaching 10^5 -fold and that of a double primer even reaching 10^9 -fold.⁴⁹ Nevertheless, the formation of the circle template at the outset of the RCA reaction is more intricate, necessitating a considerably longer reaction time than the other two amplification methods, which is not conducive to its application in the field of immediate drug resistance detection.

In conclusion, each of the three isothermal amplification methods has distinct advantages in the detection of AMR. LAMP and RCA have superior specificity and are more advantageous in the detection of drug resistance due to SNP-induced alterations in drug resistance phenotypes. Additionally, the reaction times of RPA and LAMP are shorter, which is conducive to the rapid detection of drug resistance. The reaction temperatures of RCA and RPA are more moderate, which is conducive to the concentration of microfluidic devices and the portable high-throughput detection of drug resistance.

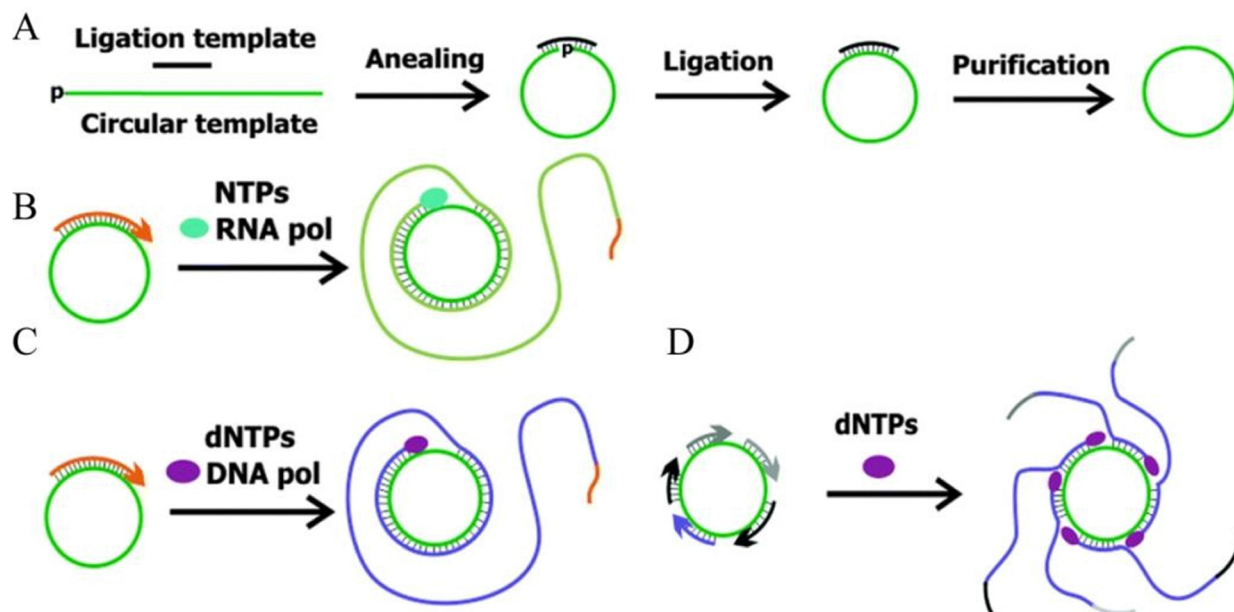


Fig. 4 Schematic illustration of the basic principles of RCA. (A) Making a DNA circle by template-mediated enzymatic ligation. (B) Linear RCA reaction to generate long ssDNA with a pre-made circle and target nucleic acids. (C) Linear RCA reaction to generate long RNA with a pre-made DNA circular template. (D) Multiprimed RCA to generate multiple copies of the RCA product from a single circle. Reproduced with permission.⁴⁶ Copyright 2014, the Royal Society of Chemistry.

However, RCA requires the lock probe to be connected to the target gene first to form a loop, which makes its integration more difficult than that of RPA. Furthermore, the connection and amplification steps of RCA are generally not carried out in the same system, which increases the difficulty of integration.

Meanwhile, in response to the need for high specificity, high throughput, portable and integrated detection of drug resistance, scientists have sought to enhance the potential of isothermal amplification through the integration of diverse technologies (Table 1).

3 The application of highly specific isothermal amplification techniques for the detection of AMR

At present, the emergence of clinical drug resistance is largely attributed to mutations in genes encoding drug targets. To illustrate, the most prevalent mutation site, *katG* 315,^{73,74} is clinically correlated with INH resistance, whereas the overwhelming majority of *rpoB* resistance-associated mutations occur in the RFP resistance-determining region.^{75,76} This mechanism of resistance generation by point mutations thus places greater demands on the specificity of resistance detection techniques. Consequently, scientists have devised various strategies to enhance the specificity of isothermal amplification techniques and rendered them more appropriate for the detection of drug resistance.

3.1 Strategy for the design of primers of base mismatches

One strategy for improving specificity is to optimize primers based on base mismatches. The fundamental premise is that,

in theory, the polymerase must be situated within the primer 3' terminal base and the template must be entirely complementary in order to facilitate an effective polymerization reaction. However, due to the polymerase's inherent variability, a number of factors can influence the outcome. In certain instances, even if the primer's 3' terminal base is not complementary to the template, the extension can still be achieved. Nevertheless, this extension is contingent and inefficient or even off-target. It is therefore possible to differentiate the mutation site specifically according to the position and number of sites that regulate base mismatches to a certain extent. In light of the aforementioned findings, primers were designed at known point mutations whose 3' end bases were mismatched in order to specifically recognize SNPs.

This strategy was currently used in LAMP and RCA. A maximum of three base mismatches is permitted. The number of mismatched primers can be one or two.^{53,54} For instance, Pakapreud Khumwan *et al.* modified the 3' and 5' ends of the FIP and partially complementary LPF primers of *katG* 315 based on LAMP to introduce base mismatches of 3 nucleotides in their sequences with the corresponding SNP positions.⁵⁴ The SNP-targeting LAMP primers are illustrated in Fig. 5(A) and (B). This modification was made in order to render the primers tunable to the SNP target amplification and to improve the specificity of the assay. The method demonstrated 100% statistical sensitivity and specificity. This base mismatch primer design strategy enables the detection of resistance mutations without the use of additional molecular components, such as probes or analyses. Furthermore, it could be paired with a hand-held potentiostat for rapid electrochemical endpoint detection, obviating the need for large-scale equipment. This

Table 1 The combination of isothermal amplification with other techniques for the detection of drug resistance genes

Isothermal technique	Coupling technology	Organism	Target	Detection time (min)	Amplification temperature (°C)	Limit of detection (LOD)	Detection methods	Ref.
LAMP	ARMS	<i>M. avium</i> strain	23S rRNA gene	65	63	—	Real-time turbidimetric	53
LAMP	Base mismatch	<i>Mycobacterium tuberculosis</i>	katG (S315T) SNP	60–75	65	5 ng μL^{-1} (genomic DNA)	Fluorescence/end-point electro-chemical detection	54
RPA	CRISPR-Cas12a	pUC-57 plasmid	sul1, qnrA-1, mcr-1, intl1	20	37	1–10 aM	Fluorescence	55
RPA	CRISPR-Cas12b	<i>E. coli</i>	mcr-1, tet(X4)	55/40	43.3/42	6.25 copies per reaction and 9 copies per reaction	Fluorescence	56
LAMP	CRISPR-Cas12a	<i>S. aureus</i>	mecA	80–85	65	1 aM	Fluorescence/LFS	57
LAMP	CRISPR-Cas12a	Plasmid	ermB	40	65	2.75×10^3 copies per μL	Lateral flow test strips and fluorescence	58
RPA	CRISPR-Cas13a	<i>Klebsiella pneumoniae</i> , <i>Escherichia coli</i>	blaKPC	<60	39	2.5 copies per μL	Fluorescence	59
RPA	CRISPR-Cas13a	<i>P. aeruginosa</i>	mexX	5 (one-tube), 40 (two-step)	39	10 aM (one-tube), 1 aM (two-step)	Fluorescence	60
LAMP	CRISPR-Cas13a	<i>K. pneumoniae</i> (OXA-48), <i>P. aeruginosa</i> (GES-5), <i>S. marcescens</i> (GES-6), <i>E. cloacae</i> complex (GES-6)	blaOXA-48, blaGES	<120	65	10^3 CFU mL^{-1} , 10^5 CFU mL^{-1} , 10^7 CFU mL^{-1}	Fluorescence	61
RPA	Lateral flow strip assay	<i>E. coli</i>	blaCTX-M, blaSHV, and blaOXA	~40	37	2.5 ng/25 μL	Naked eye	62
RPA	Lateral flow dipstick	MDR Enterobacteriaceae	mcr-1, blaNDM-1 and tet(X4)	40	37	10 copies per μL	Naked eye	63
RPA	HRP-catalyzed lateral flow immunoassay	<i>E. coli</i> , <i>K. pneumoniae</i>	blaNDM, blaKPC, mcr-1 and tet(X)	<60	41/43	10^2 CFU mL^{-1}	Naked eye	64
RPA	Surface-enhanced Raman scattering	—	blaVIM, blaKPC and blaIMP	<120	37	0.01 pM, 0.26 pM and 0.12 pM	Handheld Raman spectrometer	65
RPA	Electrochemical	<i>E. coli</i>	oxacillin resistance gene	60	39	319 CFU mL^{-1}	Amperometric signal	66
RCA	Colorimetric-photoelectrochemical	<i>E. coli</i>	tetA, tetC	—	30	17.2 aM	Quartz detection	67
LAMP	Paper-based devices	MRSA	mecA	36–43	58	10 ag per reaction (equivalent to 1 copy per reaction)	Fluorescence (portable LED transilluminator)	68
LAMP	Paper-based chip	MRSA, <i>E. coli</i>	mecA, ermC	55	64	100 copies per reaction, 285 copies per reaction	Fluorescence (hand-held light source)	69
LAMP	Centrifugal disk system	Eco, Kpn, Aba, Pae, Sau, Pmi, Ecl, Efa, Efm, <i>C. albicans</i>	CTX-M-1 group, CTX-M-9 group, KPC, NDM, IMP, OXA-23, OXA-24, OXA-48, DHA, CMY, VanA, and mecA	<90	63	1×10^3 CFU mL^{-1}	Fluorescence	70
RPA	Oil-based centrifugal microfluidic	<i>Staphylococcus aureus</i>	mecA	<20	37	<10 copies per reaction	Real-time fluorescence	71
RCA	Microfluidic disc	<i>Mycobacterium tuberculosis</i>	katG catalase peroxidase gene	90 (“one-pot” strategy), 120 (“two-pot” strategy)	50	2 pM (“one-pot” strategy), 5 pM (“two-pot” strategy)	Optomagnetic (OM)	52
LAMP	SPC chip	Lactic acid bacteria	trA, strB, vanA, vanB, tetM and tets	<120	60	1×10^4 CFU mL^{-1}	Naked eye	72

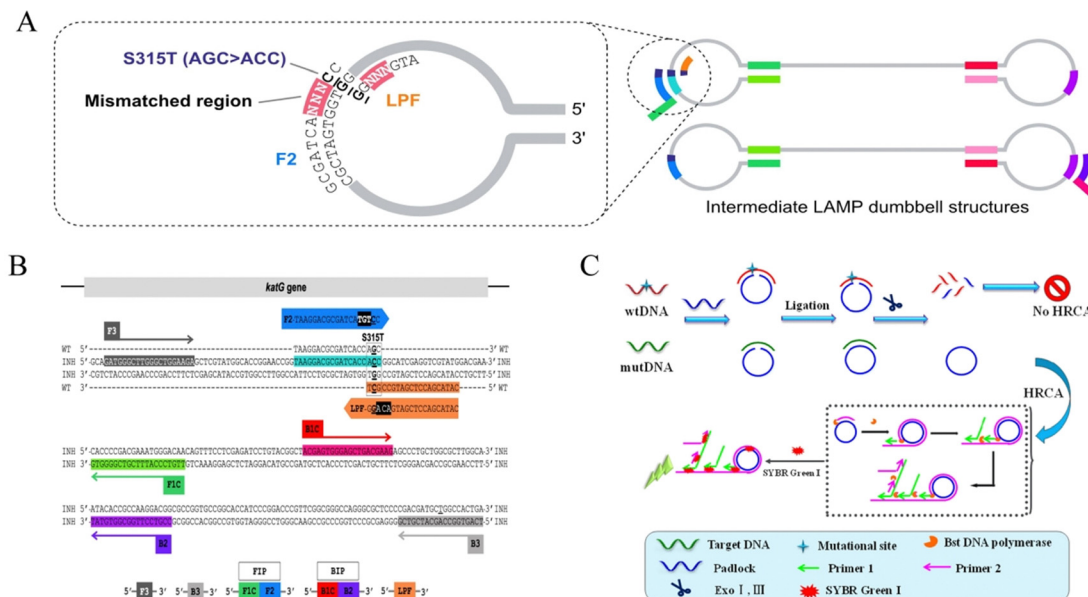


Fig. 5 (A) SNP-targeting LAMP primers. The AGC > ACC (S315T) nucleotide substitution is simultaneously targeted by the FIP and LPF primers, each individually bearing base mismatches up to 3, to help better discriminate the mutant from wild-type *katG* gene. (B) Full primer map of the chosen FIP3-LPF3 pair where the mismatch regions are adjacent to the corresponding SNP position on their respective sequences. Reproduced with permission.⁵⁴ Copyright 2022, Elsevier. (C) The principle of biosensor platform for SNP detection based on the HRCA technique. Reproduced with permission.⁷⁷ Copyright 2019, Elsevier.

is of great significance for resistance detection in resource-poor environments.

In addition to LAMP, the base mismatch strategy can also be used to design padlock probes in RCA to identify single base mutations in drug resistance genes. As shown in Fig. 5(C), the target sequence can hybridise perfectly to the designed padlock probe and form a circular padlock probe under the action of ligase to initiate the RCA reaction for subsequent extension. Wild-type sequences with only one base difference cannot form a circular padlock probe to initiate the RCA reaction.⁷⁷ To illustrate, Liu *et al.* employed a base mismatch strategy to develop padlock probes for the specific detection of single bases in genes when utilising RCA to detect *tetA* and *tetC*.⁶⁷ The primer design strategy of base mismatches represents an effective approach for enhancing the specificity of amplification. Nevertheless, this approach is pivotal for primer design and screening to ascertain specificity, and it has stringent requirements for the number and location of mismatched bases, all of which impede its application in isothermal amplification.

3.2 Strategy for conjunction with CRISPR-Cas technology

In addition to the aforementioned strategy, concatenation with CRISPR-Cas technology represents an effective strategy to enhance the specificity of drug resistance detection. The CRISPR-Cas system represents an acquired immune system that is widely found in archaea and bacteria.⁷⁸ In recent years, it has been demonstrated that class II Cas proteins possess both *cis*- and *trans*-nuclease activities.⁷⁹ The *cis*-nuclease activity enables the cleavage of specific target sequences, while the *trans*-nuclease activity allows for the cleavage of non-specific single-stranded DNA (ssDNA) or single-stranded RNA (ssRNA).⁸⁰ The *trans*-cleavage activity results in the production of a fluorescent

reporter molecule comprising ssDNA or ssRNA. Once the Cas protein-gRNA complex has formed a specific bond with the target gene, the Cas protein nuclease activity is triggered, resulting in the non-specific cleavage of the reporter molecule and the generation of a fluorescent signal. This process offers a distinctive advantage in the detection of drug resistance and is capable of discerning even subtle differences in base sequences. Currently, LAMP and RPA have been employed in conjunction with Cas12a/cas12b^{55–58} and Cas13a^{59–61} for the detection of *mcr-1*, *mecA*, OXA-48, *blaKPC* and other drug resistance genes.

In light of this property, our group devised a method for the rapid detection of *mcr-1*, which involved the coupling of the CRISPR-Cas12a system with the RPA technique. The outline of the RPA-CRISPR/Cas12a assay is illustrated in Fig. 6(A). The analytical sensitivity of our assay was 420 fg per reaction in pure *mcr-1*-positive isolates, and the threshold of this method in spiked clinical samples was down to 1.6×10^3 – 6.2×10^3 CFU mL⁻¹.⁸¹ The most commonly employed signal readout techniques are real-time PCR instrument readout and lateflow test paper method readout (Fig. 6(B)).^{56,57} To illustrate, Wang *et al.* employed a real-time PCR instrument to read out the RPA-CRISPR-Cas12a platform, which was developed for the purpose of detecting *mcr-1* and *tet(X4)*. Yet, Cao *et al.* constructed a LAMP-CRISPR-Cas12a platform for the detection of *nuc* and *mecA*, subsequently employing lateral flow test strips for the visualization of the results. The former enables the monitoring of changes in fluorescence signals in real time, making it an optimal choice for drug resistance detection in resource-rich areas. In contrast, the latter allows for the observation of amplification results through the visual detection of bands on lateral flow test paper, rendering it well-suited for resource-limited areas.

The combination of isothermal amplification technology with highly specific CRISPR/Cas enables the amplification and

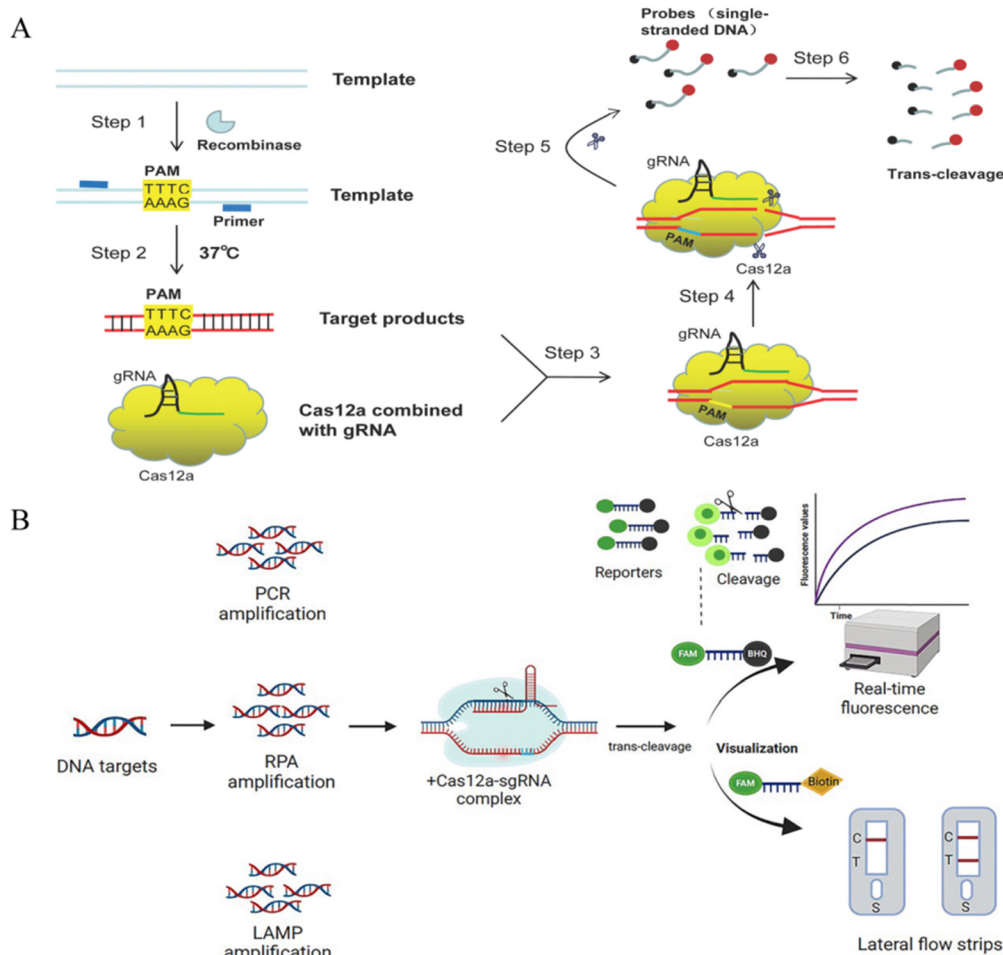


Fig. 6 (A) Outline of the RPA-CRISPR/Cas12a assay for *mcr-1* detection. (B) Schematic diagram overview of the signal readout of CRISPR/Cas12a coupled with isothermal amplification techniques. Reproduced with permission.⁵⁷ Copyright 2023, American Society for Microbiology.

enrichment of target drug resistance genes, thereby improving the detection limit. This approach is mutually complementary, allowing the full potential of isothermal amplification to be realized, and thus achieving high sensitivity and specificity for drug resistance detection. The lack of specificity of the CRISPR system for the cleavage of reporter molecules presents a significant challenge in achieving the detection of multi-drug resistance genes within the same system. In his 2018 article published in *Science*,⁸² Gootenberg JS leveraged the preference for *trans*-cutting of Cas proteins from disparate sources to achieve quadruple nucleic acid target detection. This was accomplished by employing a combination of Cas proteins from multiple sources that cleave defined reporter molecules, thereby generating reporter molecules labeled with different fluorescent moieties. Nevertheless, this multiplex amplification strategy has yet to be employed in the detection of drug resistance, likely due to the fact that the utilization of multiple enzymes markedly increases the cost, which is an impediment to its widespread adoption.

In conclusion, the strategy linked with CRISPR-Cas technology is more widely used in isothermal amplification for enhancing the specificity of drug resistance detection than base-mismatch strategies due to its strong compatibility. The combination of

isothermal amplification and CRISPR-Cas represents a highly promising technological advancement. It is anticipated that in the future, the two technologies will be integrated into a microfluidic device, facilitating the detection of multi-drug resistance with the spatial segregation of the device. This integration will optimize the benefits of isothermal amplification, enhancing its utility in the field of drug resistance detection.

4 The application of high-throughput isothermal amplification techniques for the detection of AMR

The mode of transmission of drug resistance, particularly horizontal transfer, has enabled the uninterrupted evolution of drug resistance, resulting in a characteristically diversity/multi-drug resistance phenomenon.⁸³ The conventional isothermal amplification technology is deficient in its inability to perform multiplex detection. Therefore, to achieve more accurate and rapid monitoring of AMR, there is a need for the development of high-throughput drug resistance detection methods. Currently, isothermal amplification is being employed in conjunction with a

range of detection technologies to facilitate the high-throughput detection of drug resistance genes.

4.1 Isothermal amplification technique in conjunction with lateral flow analysis

Lateral flow analysis (LFA) is one of the most commonly used high-throughput assays.^{84,85} Furthermore, multiplex LFAs, which analyze multiple targets simultaneously, are employed in conjunction with the RPA technique for the detection of drug resistance.^{62–64} Two forms of LFA are recognized: a direct method and a competitive method. Competitive methods are more frequently employed due to their superior assay specificity. For example, Tao *et al.* employed a competitive method to detect drug resistance genes.⁶⁴ The amplification primers were labelled with different markers, including digoxin, carboxytetramethylrhodamine, fluorescein isothiocyanate, and cyanine 3. This allowed the amplified fragments of the target gene to be endowed with distinctive markers, which were then captured by the corresponding antibodies with the markers immobilized in the lateral flow bands. The scheme of the cascade detection system is illustrated in Fig. 7(A) and (B). This resulted in the generation of a colorimetric signal for drug resistance detection. The developed method achieved the simultaneous detection of bla_{NDM}, bla_{KPC}, mcr-1 and tet(X) in less than one hour, with a detection limit of 10² CFU mL⁻¹. The results are typically observed visually, allowing for qualitative or semi-quantitative detection of resistance genes. It can also be used for quantitative detection. For instance, Lu *et al.* employed a test strip reader to integrate a straightforward colorimetric readout with lateral flow strips for the quantitative detection of drug resistance genes (Fig. 7(C)).⁶³

In short, the combination of LFA with isothermal amplification represents a straightforward, rapid, high-throughput and cost-effective approach to the detection of drug resistance. Furthermore, this method has the potential for on-site detection of drug resistance. Nevertheless, the high throughput is constrained by the technology of lateral immuno-flow test

strips. At present, the highest four-fold detection of drug-resistant genes has been achieved. It is anticipated that the throughput of drug-resistant detection will be enhanced in the future by optimizing the method of test strip construction.

4.2 Isothermal amplification technique in conjunction with sensing technology

Sensing technology is also one of the commonly used high-throughput detection methods. A variety of sensing technologies, including optical sensing, electrochemical sensing, and photoelectrochemical sensing coupled with isothermal amplification, have been employed for the detection of multi-drug resistance (Fig. 8(A)).^{65–67} Surface-enhanced Raman scattering (SERS) as a type of optical sensing technology, represents a highly promising readout technique for rapid diagnostic analysis. It is characterized by narrow and well-defined spectral peaks, which make it ideal for applications requiring multiplexed analysis.⁸⁶ Currently, it has been employed in conjunction with isothermal amplification technique for the high-throughput detection of ARGs. For instance, Hassanain *et al.* successfully combined a SERS assay with solid-phase RPA for the detection of three resistant carbapenemase genes (VIM, KPC, and IMP) using functionalized Au-NPs labelled with different Raman reporter groups.⁶⁵ A schematic illustration for the dual ELONA/SERS-RPA sensing platform for AMR detection is provided in Fig. 8(B). Furthermore, the assay could be coupled with a handheld Raman spectrometer, thereby facilitating on-site analysis. The SERS sensing platform for multi-drug resistance testing has been shown to possess excellent capabilities for the real-time monitoring of AMR in field settings, with regard to sensitivity, portability and the speed of analysis.

In essence, all of the aforementioned high-throughput detection strategies are founded upon the labelling of primers for a multitude of drug resistance genes, which are subsequently translated into a variety of signals for detection. Nevertheless, the capacity for multiplex detection of these strategies remains constrained. On the one

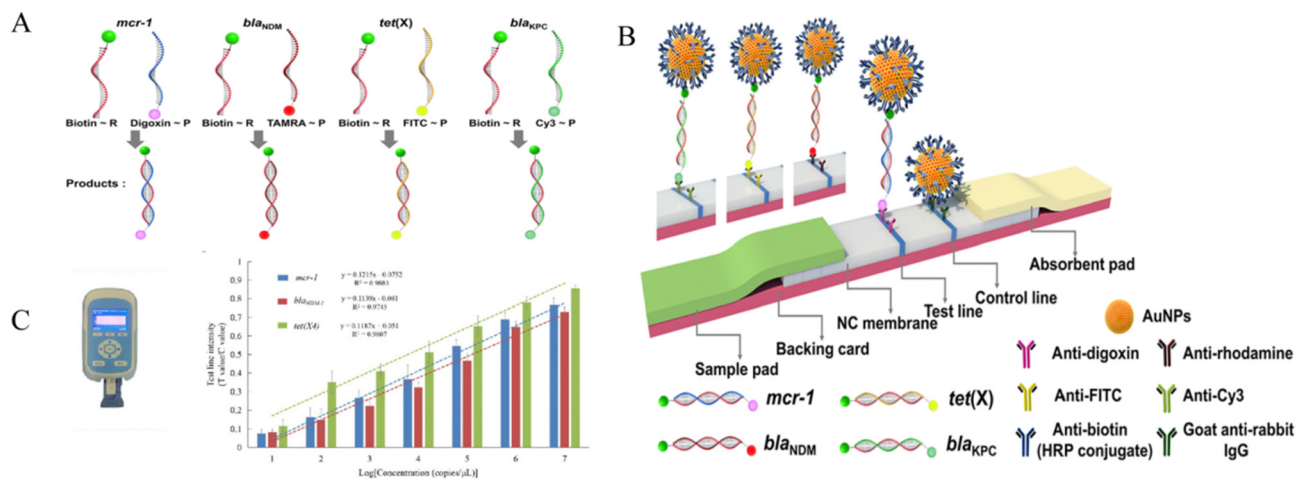


Fig. 7 (A) Scheme of the cascade detection system. Schematic illustration of RPA. (B) HRP-catalyzed LFIA. Reproduced with permission.⁶⁴ Copyright 2023, Basel, Switzerland. (C) Quantitative analysis of RPA-LFD. The standard linear equation and correlation coefficient (R^2) between the T/C value and the logarithm of the copy number of target DNA (copies per μL). Reproduced with permission.⁶³ Copyright 2022, Frontiers.

Highlight

hand, it is because isothermal amplification technology itself is difficult in primer design and not infallible. And the generation of primer dimers between multiple primers may also interfere with the amplification process, resulting in weak or even no detection signal. On the other hand, the selection of markers is difficult. There should be no signal interference between different markers. Furthermore, Sun *et al.* constructed a multivariate real-time fluorescent probe for Salmonella typing by assembling a strand-swapped three-way junction (3WJ) structure. This also provides an additional option for achieving high-throughput drug resistance detection in addition to modified primers.⁸⁷ Lastly, it is anticipated that future developments in primer design will result in further optimization and the creation of specialized primer design software for isothermal amplification. This will facilitate the application of this technique in the detection of multi-drug resistance.

5. The application of integrated isothermal amplification technology for the detection of AMR

The prevalence of drug-resistant genes in the environment and the uneven distribution of resources across regions have created a pressing need for the development of efficient drug-resistance detection methods.⁸⁸ These methods must be able to meet the challenges of on-site testing, requiring minimal equipment and resources, and offering multiplexed capabilities. They must also be suitable for use in resource-limited settings. At present, there is no testing method that meets all the desire requests. Microfluidics, a technology that integrates the entire laboratory analysis system into a single microchip,⁸⁹ offers a promising avenue for achieving this objective, leveraging the advantages of miniaturization and integration.

5.1 Isothermal amplification technique in conjunction with a capillary force-driven microfluidic-based device

Paper-based microfluidics exhibits favorable characteristics, including good biocompatibility, low cost and ease of mass manufacturing.⁹⁰ When combined with convenient isothermal

amplification techniques, it can replace the majority of instruments traditionally used for ARG amplification assays. Furthermore, these microarrays can be handled by non-specialists with a minimum number of steps, which presents a potential avenue for immediate on-site detection of AMR. A range of paper-based fluorescent LAMPs have been developed for the purpose of detecting drug resistance.^{68,69} For example, Choopara *et al.* constructed a paper-based fluorescent LAMP by immobilizing primers on a paper base based on the biotin–streptavidin-system and incubating with SYBR Green I.⁶⁸ A schematic of a paper-based LAMP device is illustrated in Fig. 9(A). This method was straightforward for the user, yields rapid results in under an hour, and had an ultra-sensitive detection limit (as low as one copy of *mecA*). Nevertheless, it has been demonstrated that the label-free nature of the insertion dye SYBR Green I leads to a considerable number of false positives when employed as a readout for LAMP results. In addition to utilizing SYBR Green I as a readout, Li *et al.* proposed the use of a “lightswitch” molecular probe (*i.e.*, $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$) as a readout modality for the detection of drug resistance by integrating the LAMP assay with it on a paper base.⁶⁹ The principle for detection was based on the evidence that in aqueous solution, the fluorescence of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ is quenched *via* the protonation of N atoms. Upon binding with double-stranded DNA, the planar phenazine ligand interacts with the base pairs in the major groove of DNA, protecting the N atoms in phenazine. This results in a change in the microenvironment of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$, increasing the population of the luminescent state and leading to intense fluorescence emission at 620 nm. Compared to SYBR Green I as a readout of amplification results, the “light switch” molecular probe has higher sensitivity and lower background interference.

In general, isothermal amplification technology based on capillary force-driven microfluidic devices is more portable and convenient for drug resistance detection with fewer operational steps. An additional concept for an assay that integrates amplification with paper-based microfluidics is to utilize existing test strips, such as those employed for pregnancy tests, to transform the identification of pathogen nucleic acids through an amplification reaction into the detection of HCG, thereby creating a portable assay. It is anticipated that this type of assay will be

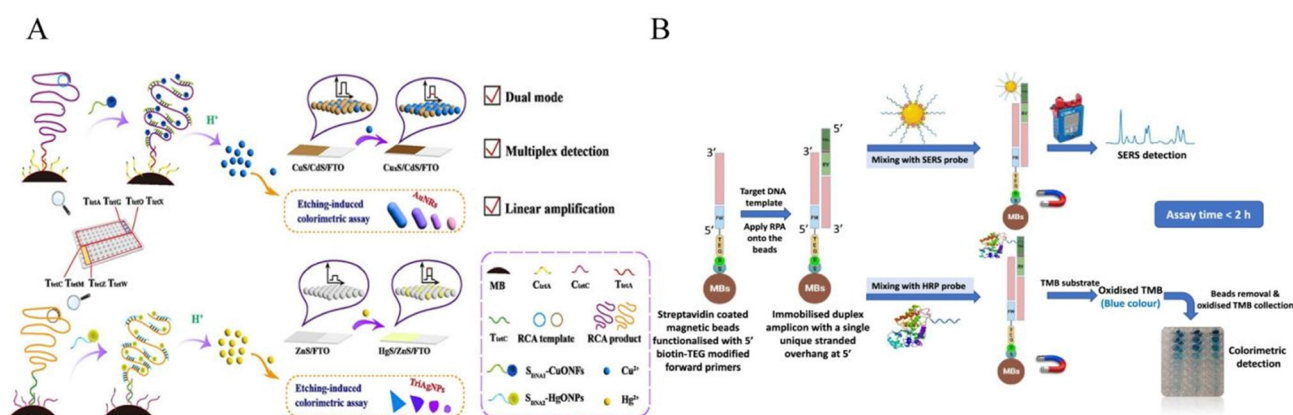


Fig. 8 (A) Principle for visual/PEC dual-mode bioassay using an RCA strategy toward multiplex ARG detection. (B) A schematic illustration of the dual ELONA/SERS-RPA sensing platform for AMR detection. Reproduced with permission.^{66,78} Copyright 2024, American Chemical Society.

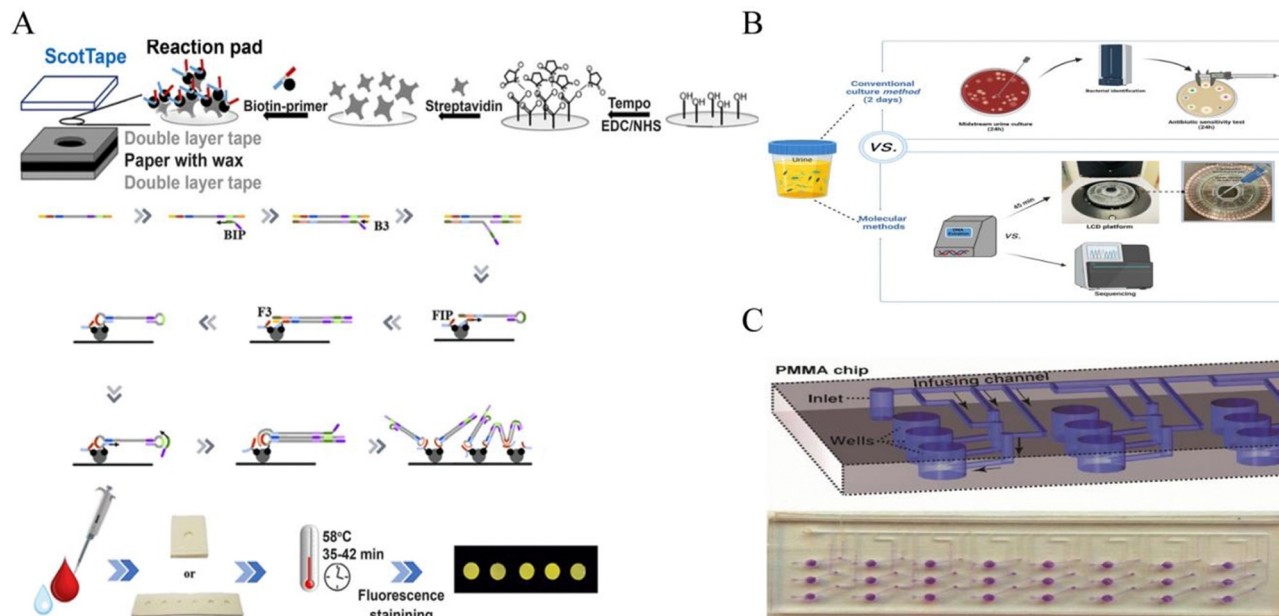


Fig. 9 (A) Schematic of a paper-based LAMP device. Design of the device and MRSA_FIP (biotinylated) immobilization, LAMP reaction steps on the paper, and overview steps for paper-based LAMP device usage. Reproduced with permission.⁶⁸ Copyright 2021, American Chemical Society. (B) The specifics of the entire procedure. Reproduced with permission.⁷⁰ Copyright 2023, Frontiers. (C) Schematic of the SPC chip. The structure of the SPC chip included an inlet, infusing channels, and reaction wells. A violet solution was successfully loaded and isolated into individual wells. Reproduced with permission.⁷² Copyright 2020, Springer.

applicable in the future for the detection of drug resistance genes.⁹¹ Furthermore, it has the potential to be applied for the immediate detection of multiple drug resistance, especially under resource-limited conditions.

5.2 Isothermal amplification technique in conjunction with a centrifugal force-driven microfluidic-based device

Although paper-based microfluidic devices are not reliant on external equipment, their open assay environment is susceptible to cross-contamination between samples, which can result in false-positive outcomes. Centrifugal force-driven microfluidic devices, which utilize centrifugal force, represent an effective solution to this issue. For instance, N. Chen *et al.* successfully achieved the detection of a wide range of bacteria and ARGs in clinical urinary tract infections by using a pre-gel method with buffer, dye and specific LAMP primers preloaded in centrifuge discs.⁷⁰ The specifics of the entire procedure are illustrated in Fig. 9(B). The method demonstrated satisfactory concordance with the conventional culture-based approach, exhibited high accuracy, and fulfilled the requisite criteria for expeditious diagnosis. In addition to LAMP, RPA and RCA can also be concentrated into a centrifugal force-driven microfluidic device for the detection of AMR.^{52,71} However, the use of centrifugal force-driven microfluidic devices is contingent upon the availability of centrifugal equipment, which is suboptimal for the on-site analysis of AMR.

5.3 Isothermal amplification technique in conjunction with a pressure-driven microfluidic-based device

The advent and evolution of pressure-driven microfluidic devices offer a potential solution to this issue.^{92,93} The vacuum-negative-pressure structure of the auto-sampling method is devoid of

instrumentation and allows for closed detection, thereby reducing the likelihood of product cross-contamination. For example, in the case of this, Jin *et al.* preloaded LAMP primers for six drug-resistant genes on the chip and employed self-priming compartmentalization technology to complete the segregation,⁷² thereby avoiding mutual interference and cross-contamination between primers (Fig. 9(C)). This approach enabled the high-throughput identification of drug-resistant genes within two hours, with a limit of detection of 10^3 CFU mL⁻¹, which was in line with that of tubular LAMP. Despite the microarray's capacity for the multiplex detection of drug resistance genes, its sensitivity remains limited. Furthermore, the auto-sampling aspect of the vacuum-negative structure presents a challenge in integrating nucleic acid extraction of samples on the microarray.

In summary, microfluidic devices utilizing diverse driving forces are employed in conjunction with isothermal amplification technology, which streamlines operational procedures, minimizes the quantity of reagents required, and facilitates the portable and immediate detection of drug resistance to a certain extent. Nevertheless, the degree of integration remains inadequate, and the extraction, amplification and product detection of drug-resistant genes cannot be conducted in a single system. It is thus evident that the construction of isothermal amplification microarrays integrating nucleic acid extraction, amplification and detection represents a prospective direction for the advancement of rapid detection of AMR.

6 Summary and outlook

Isothermal amplification, as an emerging molecular biology technique, is a promising rapid field technique due to its mild

Highlight

reaction conditions that offer unique advantages in the detection of AMR. In practice, particularly in situations where resources are limited, the utilization of isothermal amplification techniques for the expeditious detection of ARGs in the field, in comparison to laboratory-based testing, has the effect of compromising the overall performance, particularly in terms of sensitivity. Furthermore, numerous challenges exist, including the storage of amplification reagents, the multitude of upstream and downstream operational steps, and the ease of signal readout devices. As microfluidics and isothermal amplification technology continue to evolve, the technical challenges associated with integrating isothermal amplification with upstream and downstream operations can be addressed by optimizing the amplification reaction system, the detection method, as well as the microfluidic sampling method and the construction method. It is anticipated that microfluidics will evolve to offer enhanced quantitative performance, reduced instrument dependence, and isothermal amplification for the analysis of unknown resistance genes in the near future. Furthermore, the immediate detection of AMR is expected to advance towards integrated, rapid, high-throughput, and multiplex screening. The sophisticated image acquisition and analysis capabilities of smartphones offer an effective and intelligent solution for the detection of isothermal amplification microarrays, which is conducive to the widespread use of immediate drug resistance detection.

Data availability

We have written this review article based on the published articles. No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported by the National Nature Science Foundation of China (grant no. 22276066).

References

- I. N. Okeke, M. E. A. de Kraker, T. P. Van Boeckel, C. K. Kumar, H. Schmitt, A. C. Gales, S. Bertagnolio, M. Sharland and R. Laxminarayan, *Lancet*, 2024, **403**, 2426–2438.
- C. J. L. Murray and Resistance Collaborators Antimicrobial, *Lancet*, 2022, **399**, 629–655.
- S. P. Djordjevic, V. M. Jarocki, T. Seemann, M. L. Cummins, A. E. Watt, B. Drigo, E. R. Wyrsh, C. J. Reid, E. Donner and B. P. Howden, *Nat. Rev. Genet.*, 2024, **25**, 142–157.
- A. Milobedzka, C. Ferreira, I. Vaz-Moreira, D. Calderón-Franco, A. Gorecki, S. Purkrtova, B. Jan, L. Dziewit, C. M. Singleton, P. H. Nielsen, D. G. Weissbrodt and C. M. Manaia, *J. Hazard. Mater.*, 2022, **424**, 127407.
- S. Chi, W. Xu and Y. Han, *Sci. Total Environ.*, 2022, **853**, 158667.
- B. Ouyang, C. Yang, Z. Lv, B. Chen, L. Tong and J. Shi, *Environ. Int.*, 2024, **191**, 108989.
- Z. Zhang, Q. Zhang, T. Wang, N. Xu, T. Lu, W. Hong, J. Penuelas, M. Gillings, M. Wang, W. Gao and H. Qian, *Nat. Commun.*, 2022, **13**, 1553.
- B. Wang, J. Xu, Y. Wang, E. Stirling, K. Zhao, C. Lu, X. Tan, D. Kong, Q. Yan, Z. He, Y. Ruan and B. Ma, *Adv. Sci.*, 2023, **10**, e2301980.
- V. Kandavalli, P. Karempudi, J. Larsson and J. Elf, *Nat. Commun.*, 2022, **13**, 6215.
- Z. Zhao, K. Zhang, N. Wu, W. Li, W. Xu, Y. Zhang and Z. Niu, *Environ. Int.*, 2020, **135**, 105385.
- L. Wang, Z. Li, C. Y. Tay, B. J. Marshall and B. Gu, *Lancet Microbe*, 2024, **5**, e512–e513.
- E. Lancaster, R. Winston, J. Martin and J. Lee, *Water Res.*, 2024, **259**, 121818.
- H. Sidhu, H. S. Bae, A. Ogram, G. O'Connor and F. Yu, *Appl. Environ. Microbiol.*, 2021, **87**, e0037321.
- C. Hu, S. Kalsi, I. Zeimpekis, K. Sun, P. Ashburn, C. Turner, J. M. Sutton and H. Morgan, *Biosens. Bioelectron.*, 2017, **96**, 281–287.
- M. M. Hassan, A. H. M. van Vliet, O. Higgins, L. P. Burke, A. Chueiri, L. O'Connor, D. Morris, T. J. Smith and R. M. La Ragione, *Microb. Biotechnol.*, 2023, **16**, 977–989.
- M. Nakano, S. Kalsi and H. Morgan, *Biosens. Bioelectron.*, 2018, **117**, 583–589.
- Y. Xiang, K. Deng, H. Xia, C. Yao, Q. Chen, L. Zhang, Z. Liu and W. Fu, *Biosens. Bioelectron.*, 2013, **49**, 442–449.
- S. Liu, G. Huang, Y. Gong, X. Jin, Y. Meng, Y. Peng, J. Zhao, X. Li and Q. Li, *Burns Trauma*, 2020, **8**, tkaa026.
- J. Tao, D. Liu, J. Xiong, L. Dou, W. Zhai, R. Zhang, Y. Wang, J. Shen and K. Wen, *Microbiol. Spectrum*, 2022, **10**, e0334422.
- M. Kühnemund, I. Hernández-Neuta, M. I. Sharif, M. Cornaglia, M. A. M. Gijs and M. Nilsson, *Nucleic Acids Res.*, 2017, **45**, e59.
- I. M. Lobato and C. K. O'Sullivan, *TrAC, Trends Anal. Chem.*, 2018, **98**, 19–35.
- W. Yin, J. Zhuang, J. Li, L. Xia, K. Hu, J. Yin and Y. Mu, *Small*, 2023, **19**, e2303398.
- R. R. G. Soares, N. Madaboosi and M. Nilsson, *Acc. Chem. Res.*, 2021, **54**, 3979–3990.
- E. A. Pumford, J. Lu, I. Spaczai, M. E. Prasetyo, E. M. Zheng, H. Zhang and D. T. Kamei, *Biosens. Bioelectron.*, 2020, **170**, 112674.
- J. Song, H. Y. Kim, S. Kim, Y. Jung and H. G. Park, *Biosens. Bioelectron.*, 2021, **178**, 113051.
- B. Veigas, P. Pedrosa, I. Couto, M. Viveiros and P. V. Baptista, *J. Nanobiotechnol.*, 2013, **11**, 38.
- X. Zhao, Y. Zeng, B. Yan, Y. Liu, Y. Qian, A. Zhu, Y. Zhao, X. Zhang, C. Zhang and Z. Wan, *Microbiol. Spectrum*, 2024, **12**, e0413323.
- K. Nawattanapaiboon, W. Kiatpathomchai, P. Santanirand, A. Vongsakulyanon, R. Amarit, A. Somboonkaew, B. Sutapun and T. Sriksirin, *Biosens. Bioelectron.*, 2015, **74**, 335–340.
- J. Rodriguez-Manzano, N. Moser, K. Malpartida-Cardenas, A. Moniri, L. Fisarova, I. Pennisi, A. Boonyasiri, E. Jauneikaite, A. Abdolrasouli, J. A. Otter, F. Bolt, F. Davies, X. Didelot, A. Holmes and P. Georgiou, *Sci. Rep.*, 2020, **10**, 8448.
- H. W. Li, J. Y. Mao, C. W. Lien, C. K. Wang, J. Y. Lai, R. P. Mandal, H. T. Chang, L. Chang, D. H. Ma and C. C. Huang, *J. Mater. Chem. B*, 2020, **8**, 3506–3512.
- T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino and T. Hase, *Nucleic Acids Res.*, 2000, **28**, E63.
- J. J. Li, C. Xiong, Y. Liu, J. S. Liang and X. W. Zhou, *Front. Plant Sci.*, 2016, **7**, 1956.
- K. Nagamine, T. Hase and T. Notomi, *Mol. Cell. Probes*, 2002, **16**, 223–229.
- G. Yu, L. Chen, C. W. Lin, B. Li, H. Cui, S. Chen, J. Miao, H. Bian, D. Chen and Y. Deng, *Biol. Res.*, 2014, **47**, 53.
- G. Chen, L. Chen, S. Lin, C. Yang, H. Liang, K. Huang, Z. Guo and F. Lv, *BMC Microbiol.*, 2023, **23**, 329.
- Z. Xu, L. Li, J. Chu, B. M. Peters, M. L. Harris, B. Li, L. Shi and M. E. Shirliff, *Food Res. Int.*, 2012, **47**, 166–173.
- Y. S. Jiang, S. Bhadra, B. Li, Y. R. Wu, J. N. Milligan and A. D. Ellington, *Anal. Chem.*, 2015, **87**, 3314–3320.
- O. Piepenburg, C. H. Williams, D. L. Stemple and N. A. Armes, *PLoS Biol.*, 2006, **4**, e204.
- I. M. Lobato and C. K. O'Sullivan, *TrAC, Trends Anal. Chem.*, 2018, **98**, 19–35.

- 40 M. M. Nelson, C. L. Waldron and J. R. Bracht, *BMC Infect. Dis.*, 2019, **19**, 144.
- 41 C. L. Johnson, M. A. Setterfield, W. A. Hassanain, A. Wipat, M. Pocock, K. Faulds, D. Graham and N. Keegan, *Analyst*, 2024, **149**, 1527–1536.
- 42 A. K. S. Ayfan, J. Macdonald, P. N. A. Harris, C. Heney, D. L. Paterson, E. Trembizki, C. Y. T. Wang, D. M. Whiley, H. M. Zowawi and A. D. Irwin, *Eur. J. Clin. Microbiol. Infect. Dis.*, 2021, **40**, 2447–2453.
- 43 M. A. Abdou Mohamed, H. N. Kozlowski, J. Kim, K. Zagorovsky, M. Kantor, J. J. Feld, S. Mubareka, T. Mazzulli and W. C. W. Chan, *ACS Nano*, 2021, **15**, 9379–9390.
- 44 A. Srisrattakarn, P. Tippayawat, A. Chanawong, R. Tavichakomtrakool, J. Daduang, L. Wonglakorn, P. Sooksongsoontorn and A. Lulitanond, *World J. Microbiol. Biotechnol.*, 2020, **36**, 162.
- 45 A. Fire and S. Q. Xu, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 4641–4645.
- 46 M. M. Ali, F. Li, Z. Zhang, K. Zhang, D. K. Kang, J. A. Ankrum, X. C. Le and W. Zhao, *Chem. Soc. Rev.*, 2014, **43**, 3324–3341.
- 47 F. Zhu, H. Zhang, R. Wu, Y. Lu, J. Wang, R. A. T. S. G. N. Zhu, Z. Zhang and J. Tang, *Biosens. Bioelectron.*, 2024, **250**, 116057.
- 48 G. Jin, C. Wang, L. Yang, X. Li, L. Guo, B. Qiu, Z. Lin and G. Chen, *Biosens. Bioelectron.*, 2015, **63**, 166–171.
- 49 J. Dong, Z. Zeng, R. Sun, X. Zhang, Z. Cheng, C. Chen and Q. Zhu, *Biosens. Bioelectron.*, 2021, **192**, 113508.
- 50 X. Chen, B. Wang, W. Yang, F. Kong, C. Li, Z. Sun, P. Jelfs and G. L. Gilbert, *J. Clin. Microbiol.*, 2014, **52**, 1540–1548.
- 51 S. J. Mun, W. Jang, H. S. Park, Y. J. Lim, T. J. Yang and K. W. Bong, *Biosens. Bioelectron.*, 2023, **241**, 115670.
- 52 G. A. S. Minero, M. Bagnasco, J. Fock, B. Tian, F. Garbarino and M. F. Hansen, *Anal. Bioanal. Chem.*, 2020, **412**, 2705–2710.
- 53 T. Inagaki, S. Asahi, K. Ogawa, T. Nakagawa, T. Ohkura, Y. Osada, T. Nikai, K. Yamada, T. Yagi and K. Uchiya, *Microbiol. Spectrum*, 2024, **12**, e0233923.
- 54 P. Khumwan, S. Pengpanich, J. Kampeera, W. Kamsong, C. Karuwan, A. Sappat, P. Srilohasin, A. Chaiprasert, A. Tuantranont and W. Kiatpathomchai, *Microchem. J.*, 2022, **175**, 107108.
- 55 Z. Cheng, X. Luo, D. Liu, J. Han, H. Wang, D. Min and H. Yu, *Environ. Sci. Technol.*, 2024, **58**, 9636–9645.
- 56 Y. Wang, H. Chen, Q. Pan, J. Wang, X. Jiao and Y. Zhang, *Appl. Microbiol. Biotechnol.*, 2024, **108**, 345.
- 57 X. Cao, Y. Chang, C. Tao, S. Chen, Q. Lin, C. Ling, S. Huang and H. Zhang, *Microbiol. Spectrum*, 2023, **11**, e0487022.
- 58 K. Mao, H. Zhang, F. Ran, H. Cao, R. Feng, W. Du, X. Li and Z. Yang, *J. Hazard. Mater.*, 2024, **462**, 132793.
- 59 M. Liang, B. Xiao, L. Chen, X. Huang, J. Li, Z. Kuang, X. Chen, X. Huang, Z. Sun and L. Li, *Curr. Microbiol.*, 2023, **80**, 352.
- 60 X. Zhu, Y. Wang, S. Li, R. Peng, X. Wen, H. Peng, Q. Shi, G. Zhou, X. Xie and J. Wang, *Front. Microbiol.*, 2024, **15**, 1341179.
- 61 C. Ortiz-Cartagena, D. Pablo-Marcos, L. Fernández-García, L. Blasco, O. Pacios, I. Bleriot, M. Siller, M. López, J. Fernández, B. Aracil, P. A. Fraile-Ribot, S. García-Fernández, F. Fernández-Cuenca, M. Hernández-García, R. Cantón, J. Calvo-Montes and M. Tomás, *Microbiol. Spectrum*, 2023, **11**, e0132923.
- 62 S. Kanokudom, T. Assawakongkarat, Y. Akeda, P. Rattawongjirakul, R. Chuanchuen and N. Chaichanawongsaroj, *PLoS One*, 2021, **16**, e0248536.
- 63 C. Z. Lu, J. W. Wang, L. M. Pan, X. Y. Gu, W. J. Lu, D. Chen, C. Zhang, Q. Ye, C. G. Xiao and P. P. Liu, *Front. Microbiol.*, 2023, **13**, 1062577.
- 64 J. Tao, D. J. Liu, J. C. Xiong, W. C. Shan, L. A. Dou, W. S. Zhai, Y. Wang, J. Z. Shen and K. Wen, *Int. J. Mol. Sci.*, 2023, **24**, 6784.
- 65 W. A. Hassanain, C. L. Johnson, K. Faulds, N. Keegan and D. Graham, *Anal. Chem.*, 2024, **96**, 12093–12101.
- 66 A. Butterworth, P. Pratibha, A. Marx and D. K. Corrigan, *ACS Sens.*, 2021, **6**, 3773–3780.
- 67 L. Liu, Q. Yao, F. Jiang, Z. Cai, M. Meng, H. Sun, L. Zhang and J. Gong, *Anal. Chem.*, 2024, **96**, 2939–2948.
- 68 I. Choopara, A. Suea-Ngam, Y. Teethaisong, P. D. Howes, M. Schmelcher, A. Leelahavanichkul, S. Thunyaharn, D. Wongsawaeng, A. J. deMello, D. Dean and N. Somboonna, *ACS Sens.*, 2021, **6**, 742–751.
- 69 B. Li, X. Zhou, H. Liu, H. Deng, R. Huang and D. Xing, *ACS Appl. Mater. Interfaces*, 2018, **10**, 4494–4501.
- 70 N. Chen, G. Li, Y. Si, Y. Ye, T. Zhang, D. Chi, W. Zhang, L. Pan, G. Qu, Y. Lu, M. Zong, G. Sui and L. Fan, *Front. Microbiol.*, 2023, **14**, 1157403.
- 71 S. Lutz, P. Weber, M. Focke, B. Faltin, J. Hoffmann, C. Müller, D. Mark, G. Roth, P. Munday, N. Armes, O. Piepenburg, R. Zengerle and F. von Stetten, *Lab Chip*, 2010, **10**, 887–893.
- 72 Z. Jin, G. Ding, G. Yang, G. Li, W. Zhang, L. Yang and W. Li, *Appl. Microbiol. Biotechnol.*, 2020, **104**, 6375–6383.
- 73 L. Liu, F. Jiang, L. Chen, B. Zhao, J. Dong, L. Sun, Y. Zhu, B. Liu, Y. Zhou, J. Yang, Y. Zhao, Q. Jin and X. Zhang, *Emerging Microbes Infect.*, 2018, **7**, 183.
- 74 E. Rivière, M. G. Whitfield, J. Nelen, T. H. Heupink and A. Van Rie, *Clin. Microbiol. Infect.*, 2020, **26**, 1332–1337.
- 75 I. Barilar, T. Fernando, C. Utpatel, C. M. Madeira, B. José, C. Mutaquilha, K. Kranzer, T. Niemann, N. Ismael, L. de Araujo, T. Wirth, S. Niemann and S. Viegas, *Lancet Infect. Dis.*, 2024, **24**, 297–307.
- 76 Y. Li, X. Kong, W. Song, Y. Li, Y. Li, W. Fang, J. Yang, C. Yu, H. Li and Y. Liu, *Emerging Microbes Infect.*, 2024, **13**, 2294858.
- 77 X. H. Li, X. L. Zhang, J. Wu, N. Lin, W. M. Sun, M. Chen, Q. S. Ou and Z. Y. Lin, *Talanta*, 2019, **191**, 277–282.
- 78 A. Marchfelder, *RNA Biol.*, 2013, **10**, 655–658.
- 79 K. S. Makarova, Y. I. Wolf, J. Iranzo, S. A. Shmakov, O. S. Alkhnbashi, S. J. J. Brouns, E. Charpentier, D. Cheng, D. H. Haft, P. Horvath, S. Moineau, F. J. M. Mojica, D. Scott, S. A. Shah, V. Siksnys, M. P. Terns, Č. Venclovas, M. F. White, A. F. Yakunin, W. Yan, F. Zhang, R. A. Garrett, R. Backofen, J. Oost, R. Barrangou and E. V. Koonin, *Nat. Rev. Microbiol.*, 2020, **18**, 67–83.
- 80 S. R. Rananaware, E. K. Vesco, G. M. Shoemaker, S. S. Anekar, L. S. W. Sandoval, K. S. Meister, N. C. Macaluso, L. T. Nguyen and P. K. Jain, *Nat. Commun.*, 2023, **14**, 5409.
- 81 L. Gong, Z. Jin, E. Liu, F. Tang, F. Yuan, J. Liang, Y. Wang, X. Liu and Y. Wang, *Microbiol. Spectrum*, 2022, **10**, e0188422.
- 82 J. S. Gootenberg, O. O. Abudayyeh, M. J. Kellner, J. Joung, J. J. Collins and F. Zhang, *Science*, 2018, **360**, 439–444.
- 83 C. P. F. Domingues, J. S. Rebelo, F. Ionisio and T. Nogueira, *Int. J. Mol. Sci.*, 2023, **24**, 11438.
- 84 C. Wang, C. Wang, J. Li, Z. Tu, B. Gu and S. Wang, *Biosens. Bioelectron.*, 2022, **214**, 114525.
- 85 M. M. Chang, M. E. Natoli, A. F. Wilkinson, V. N. Tubman, G. E. Airewele and R. R. Richards-Kortum, *Lab Chip*, 2024, **24**, 4115–4127.
- 86 S. Laing, K. Gracie and K. Faulds, *Chem. Soc. Rev.*, 2016, **45**, 1901–1918.
- 87 Y. Sun, S. Zhang, L. Qi, X. Zhang, M. Yang, Z. Guo, Z. Wang and Y. Du, *Anal. Chem.*, 2023, **95**, 17808–17817.
- 88 X. P. Guo, Y. Yang, D. P. Lu, Z. S. Niu, J. N. Feng, Y. R. Chen, F. Y. Tou, E. Garner, J. Xu, M. Liu and M. F. Hochella, *Water Res.*, 2018, **129**, 277–286.
- 89 W. Postek and P. Garstecki, *Acc. Chem. Res.*, 2022, **55**, 605–615.
- 90 S. B. Somvanshi, A. M. Ulloa, M. Zhao, Q. Liang, A. K. Barui, A. Lucas, K. M. Jadhav, J. P. Allebach and L. A. Stanciu, *Biosens. Bioelectron.*, 2022, **207**, 114214.
- 91 L. Qi, M. Yang, D. Chang, W. Zhao, S. Zhang, Y. Du and Y. Li, *Angew. Chem., Int. Ed.*, 2021, **60**, 24823–24827.
- 92 M. V. Chinnamani, A. Hanif, P. K. Kannan, S. Kaushal, M. J. Sultan and N. E. Lee, *Biosens. Bioelectron.*, 2023, **237**, 115468.
- 93 Z. Xu, D. Chen, T. Li, J. Yan, J. Zhu, T. He, R. Hu, Y. Li, Y. Yang and M. Liu, *Nat. Commun.*, 2022, **13**, 6480.