



Loop-Mediated Isothermal Amplification–Based Microfluidic Platforms for the Detection of Viral Infections

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Abstract

Purpose of Review Easy-to-use, fast, and accurate virus detection method is essential for patient management and epidemic surveillance, especially during severe pandemics. Loop-mediated isothermal amplification (LAMP) on a microfluidic platform is suitable for detecting infectious viruses, regardless of the availability of medical resources. The purpose of this review is to introduce LAMP-based microfluidic devices for virus detection, including their detection principles, methods, and application.

Recent Findings Facing the uncontrolled spread of viruses, the large-scale deployment of LAMP-based microfluidic platforms at the grassroots level can help expand the coverage of nucleic acid testing and shorten the time to obtain test reports. Microfluidic chip technology is highly integrated and miniaturized, enabling precise fluid control for effective virus detection. Performing LAMP on miniaturized systems can reduce analysis time, reagent consumption and risk of sample contamination, and improve analytical performance.

Summary Compared to traditional benchtop protocols, LAMP-based microfluidic devices reduce the testing time, reagent consumption, and the risk of sample contamination. In addition to simultaneous detection of multiple target genes by special channel design, microfluidic chips can also integrate digital LAMP to achieve absolute quantification of target genes.

Keywords Point-of-care · Microfluidic platform · LAMP · Detection strategy · Virus detection

Introduction

Emerging and re-emerging infectious viruses pose a critical threat to global public health [1]. Therefore, it is necessary to screen for carriers in the population via laboratory diagnostic tests to inhibit the transmission chain of the virus. Serological tests and nucleic acid detection techniques are more suitable for viral infection screening in large populations than virus isolation and culture techniques [2, 3]. Immunology-based serological tests detect antigens and

antibodies associated with viruses. Although they offer rapid detection, serological tests have a long detection window and low sensitivity, limiting their use in viral infectious diseases [4]. Polymerase chain reaction (PCR) testing is considered the gold standard for detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [5, 6]. Although the current PCR technology is still adequate for virus detection, there are some weaknesses that limit its application, such as expensive analysis equipment, long turnaround time, and dependence on specialized technicians [7, 8]. Microfluidic detection systems are new nucleic acid detection platforms, achieving the “sample-to-answer” goal by integrating sample preparation, amplification reaction, and signal detection into one miniature system. Besides, microfluidics-based assays allow viral nucleic acid testing to be performed by non-specialists outside a laboratory [9, 10]. This reduces the testing time, reagent consumption, and the risk of sample contamination, easing the testing requirements burden. Therefore, it is important to develop a fast and accurate nucleic acid testing method on a microfluidic device. However, the temperature requirement of PCR limits the design

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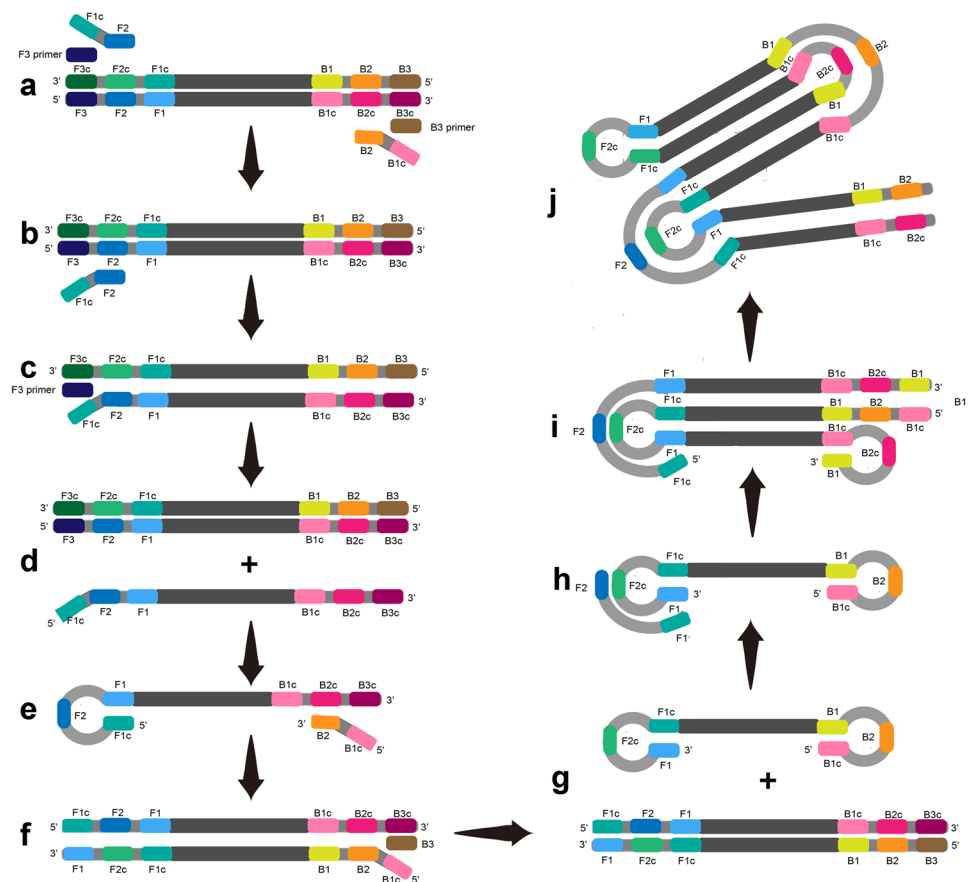
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and manufacture of the PCR-associated microfluidic devices. Isothermal amplification (IA) of nucleic acids is a promising option for efficient amplification at a constant temperature. Loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), rolling circle amplification (RCA), nucleic acid sequence–based amplification (NASBA), and helicase-dependent amplification (HDA) are the common IA approaches [11, 12]. IA can be combined with microfluidics at a constant temperature using a simple heating device compared to PCR techniques that require three temperature cycles. However, they also have some drawbacks that limit their application. For instance, non-specific amplifications cause high background noise, complex enzymes complicate the assay process, and low tolerances require an optimal reaction system. In comparison with other IA methods, LAMP has received widespread attention due to its unique specificity [13]. LAMP-based microfluidic assay platforms are emerging and have shown good potential for virus detection. This review provides a brief overview of LAMP technology for virus detection. Moreover, the amplicon analysis methods, the multiplex LAMP assay, and digital LAMP based on microfluidics are described. Finally, methods for integrating LAMP with microfluidics are discussed.

LAMP for Virus Detection

LAMP is an auto-cyclic chain substitution reaction performed at a constant temperature and synthesizes several amplification products within 1 h. LAMP reaction requires at least four primers to amplify six different target regions (Fig. 1). The primers include the forward inner primer (FIP), the backward inner primer (BIP), the forward outward primer (F3), and the backward outward primer (B3). FIP is composed of the F2 region at the 3' end and the F1c region at the 5' end; BIP is formed by the B2 region at the 3' end and the B1c region at the 5' end. The F2 region is bound to the target sequence during the non-cyclic step, then extended by the *Bst* DNA polymerase. The outer F3 primer then hybridizes to the same target strand of the F3c region, and the F3 extended strand replaces the FIP synthetic strand. A stem-loop structure is formed at the 5' end of the replacement strand due to the binding of F1c and the F1 region. The same hybridization principle is repeated in the reverse primer set bound to the chain at the 3' end, forming a dumbbell-structured chain with a stem-loop structure at both ends. Only two internal primers trigger DNA synthesis during the loop step by hybridizing with the loop on the product

Fig. 1 Graphical representation of LAMP amplification process. **a–g** Non-cyclic step. DNA with dumbbell-like structures at both ends was produced, which underwent the recycling step. **h–j** Cyclic amplification step. Exponential amplification of the original dumbbell-shaped stem-loop DNA was performed using inner primers



of the previous stage. This produces amplicons containing multiple repeats of the target and a cauliflower-like polycyclic structure [14••]. Significantly, the process of LAMP reaction can be accelerated by using two loop primers. The amplification time can be reduced to less than 30 min by the addition of loop primers [15].

Based on the genetic core material carried, viruses can be classified as DNA viruses and RNA viruses. RNA viruses are more prone to genetic mutation than DNA viruses due to their high mutation and replication rates [16, 17]. It makes it difficult to control the epidemic of infectious diseases caused by RNA viruses. In the original LAMP report, it was successfully applied to the detection of DNA virus (hepatitis B virus) by Notomi et al. [14••]. The LAMP reaction component of the type consists of six designed primers (FIP, BIP, F3, B3, and two loop primers), dNTP, betaine, KCl, $(\text{NH}_4)_2\text{SO}_4$, MgSO_4 , Tris-HCl, and *Bst* DNA polymerase. It was shown in subsequent studies that the detection of RNA viruses can also be achieved in the combined use of reverse transcriptase (RTase) and *Bst* DNA polymerase [18–20]. For instance, West Nile Virus was successfully detected using RT-LAMP by Parida et al. Turbidity changes were monitored at 17 min by incubating viral RNA with reaction components containing RTase and DNA polymerase in a single tube at 63 °C. Until now, RT-LAMP has been used for the detection of influenza A virus, zika virus (ZIKV), human immunodeficiency virus (HIV), human papillomavirus (HPV), dengue virus, SARS-CoV-2, and other viruses [21–23]. The advent of microfluidic technology has brought about the emergence of miniaturized and disposable LAMP assay chips. LAMP-based microfluidic chips using colorimetric, fluorescence, lateral flow assay, and electrochemical amplicon analysis technologies enable the detection of viruses outside the laboratory, bringing a new paradigm for timely and rapid virus screening.

Turbidity Detection

Turbidity detection is the simplest and most convenient LAMP detection method. Several pyrophosphate ions are released into the reaction system during the LAMP reaction process. The ions react with the magnesium ions to form a white magnesium pyrophosphate precipitate [24]. The white precipitate signifies the amplification of the target sequence. An ideal LAMP reaction contains a large amount of white precipitate, and turbidity changes of the reaction system can be observed without centrifugation. However, when the product is inadequate, the change in turbidity is not significant. Furthermore, it is difficult to accurately identify turbidity changes in the reaction solution, leading to the risk of missed diagnosis. Therefore, electronic analysis equipment should be used to monitor

turbidity changes and quantify the nucleic acid concentration of the virus, to improve sensitivity [25].

Colorimetric Detection

Colorimetric detection is used to determine whether an amplification reaction has occurred, as indicated by the color change of the reaction system before and after amplification. The color change is achieved by adding different indicators, and the principle varies from one indicator to another. Metal ion indicators, such as hydroxyl naphthol blue (HNB) [26] and Eriochrome black T (EBT) [27], change color through the loss of magnesium ions. For instance, HNB combines with magnesium ions at the beginning of the reaction to form a violet color. The magnesium ions and pyrophosphate ions then form a precipitate as the reaction proceeds, and HNB loses magnesium ions, thus a blue solution (Fig. 2a). Similarly, EBT turns from violet to sky blue before and after amplification (Fig. 2a) [28]. Besides, the change in calcein color from orange to fluorescent green before and after amplification has also been used to indicate LAMP reaction results [29]. The fluorescence of calcein is quenched by manganese ions before amplification, leading to an orange solution. The calcein molecule then loses the manganese ion after amplification and binds to the newly generated pyrophosphate ion, thus restoring its green fluorescence [30, 31] (Fig. 2a). In 2020, Yu et al. reported a self-dispensing Slip-Chip microfluidic device for DNA detection of HPV using slip-induced droplet LAMP with calcein. The SlipChip has a “chain of pearls” continuous microfluidic channel design activated by a simple slip step, self-distributing the fluids into individual droplets through capillary pressure-driven flow. The limit of detection (LOD) was 7×10^2 copies mL^{-1} [32]. Furthermore, DNA binding dyes, such as SYBR Green I [33] and SYTO-9 [34], can make the amplification results more observable by non-specifically binding to the double-stranded DNA. The dyes hardly emit fluorescence when they are in an unbound state in the solution, while they emit a strong fluorescent signal after binding to the double-stranded DNA [35, 36] (Fig. 2a). De Oliveira et al. reported a centrifugal polystyrene generator (PS-T) microfluidic device controlled by a fidget spinner. Visual detection with SYBR Green I was used to analyze RT-LAMP amplicons via a smartphone to capture the signal. The sensitivity validation on SARS-COV-2 clinical samples had a LOD of 10^{-3} RNA copies/reaction [37•].

The Lateral Flow Assay

The lateral flow assay (LFA) is an immunochromatographic technique that utilizes antibody capture followed by secondary antibody and presents the analytical results of LAMP

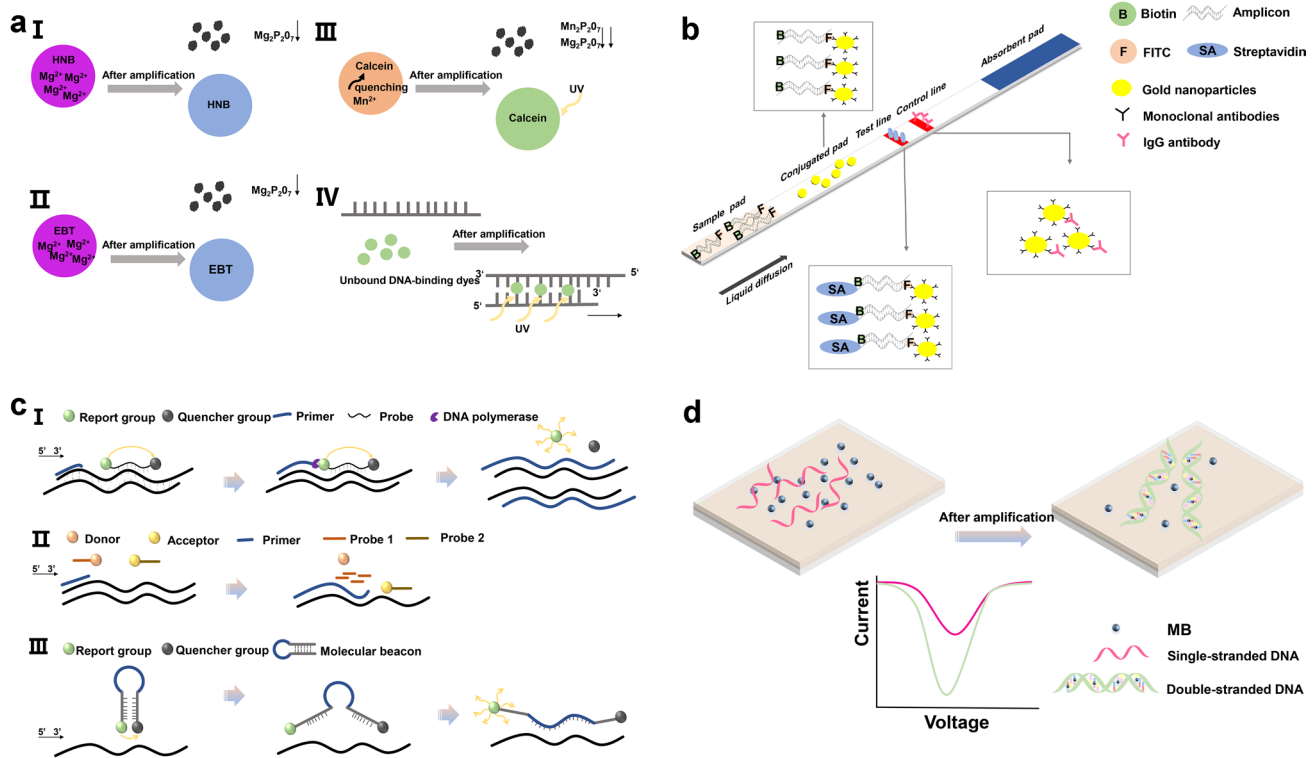


Fig. 2 Principle of the different methods for analyzing LAMP amplicons. **a** Color comparison of different dyes before and after amplification. (I) HNB indicator. The color changes from violet to blue before and after the reaction. (II) EBT indicator. The color changes from violet to blue before and after the reaction. (III) Calcein indicator. The color changes from orange to green before and after the reaction. (IV) DNA-binding dye indicators. The color changes from no fluorescence to green before and after the reaction. **b** Schematic diagram of amplicon analysis on lateral flow test strips. The sample forms a

products in a colorimetric manner on a lateral flow test strip, the results of which can be confirmed by the naked eye [38–40]. One strategy of the method is to label the primers with biotin and fluorescein isothiocyanate (FITC), respectively, and sandwich both ligands in the LAMP product. In addition, gold nanoparticles are immobilized on the conjugate pad of the lateral flow strip, while streptavidin is immobilized in the sample detection area and general IgG antibody is immobilized in the quality control area. As the labeled amplicon diffuses on the test strip, a visible detection line is formed in the sample detection zone by forming streptavidin–biotin–DNA–FITC–gold nanoparticle complex [41]. If there are no labeled amplicons, the detection line will not be visible. The quality control line is used to confirm that the results are reliable (Fig. 2b). In the case of influenza A virus detection, centrifugal microfluidic device for influenza A virus detection using LFA to analyze amplicons of RT-LAMP was reported by Jung et al. Results seen with the naked eye are obtained within 55 min after loading purified viral RNA into the device, i.e., judged by the presence

or absence of detection and quality control lines, and LOD was 10 copies/reaction [42]. For the highly sensitive LAMP reaction, opening the cap will undoubtedly increase the likelihood of sample contamination significantly. Although this assay is easy to use, inexpensive, and rapid, it is difficult to fully integrate the LAMP amplification process into the test strip. Therefore, achieving LFA and LAMP integration remains a huge challenge [43].

complex with the gold nanoparticle–antibody if it contains biotin- and FITC-labeled nucleic acid, which can be seen by the naked eye. Otherwise, the detection line cannot be seen. However, the quality control line should be seen regardless of whether there is target nucleic acid in the sample or not. Otherwise, the result is invalid. **c** Three different strategies for fluorescent probe technology: hydrolysis probes, dual probes, and molecular beacons. **d** Schematic diagram of the principle of monitoring LAMP process using MB

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Fluorescence Detection

Fluorescence detection, including fluorescent dye detection and fluorescent probe detection, is one of the most widely used nucleic acid detection methods due to its high sensitivity. This section focuses on fluorescent probe technology. A fluorescent probe is a real-time fluorescence quantitative technology based on fluorescence resonance energy transfer (FRET), a quantum phenomenon occurring between two dye molecules. The fluorescence energy is transferred when

the distance between a fluorescent group and a quenching group is close to a specific range. Moreover, the quenching group can absorb the excited fluorescence in the fluorescent group under the action of the excitation factor and does not emit fluorescence. However, after the fluorescent group, the quenching effect disappears from the quenching group, thus the fluorescence. Therefore, various real-time fluorescence quantitative technologies can be established by selecting appropriate fluorescent and quenching groups to label the nucleic acid probes [44–46]. Hydrolysis probes, dual probes, and molecular beacons are some of the primary detection strategies [47, 48] (Fig. 2c). In some cases, two specific FRET probes are designed to aim at one loop region within LAMP amplicons labeled with different functional groups. The specific probe is added to the LAMP reaction system, as the fluorescence change indicates the result of the reaction after amplification. The initial concentration of the target nucleic acid can be calculated according to the predetermined standard curve when the cycle threshold value has been obtained. For instance, Wan et al. developed a LAMP-based microfluidic system using molecular beacon DNA probes. LAMP-on-a-chip for unknown sample detection can be completed in 40 min with a detection limit of 10 copies/reaction [49].

Electrochemical Detection

Electrochemical detection can be incorporated into the POC platforms since it is fast, sensitive, inexpensive, and can be easily miniaturized [50]. Importantly, robustness of electrochemical methods allows accurate detection even in heterogeneous and impure samples [51]. Amplification was monitored by using the potential changes generated when the electroactive reagent binds to the LAMP product. Nagatani et al. developed a USB-powered portable potentiostat for semi-real-time electrochemical monitoring using the electroactive reagent methylene blue (MB) and successfully quantified influenza virus RNA by RT-LAMP [52]. The MB binds to the double-stranded DNA during the LAMP reaction, decreasing free MB concentration in the reaction system. Therefore, the peak current decreases with the reaction time (Fig. 2d). In the study by Kuangwen et al., a LAMP reaction mixture containing DNA template and MB was injected into a single-chamber chip for real-time electrochemical monitoring [50]. Besides, the use of novel nanomaterials to modify electrodes enables monitoring of the reaction system using changes in the impedance magnitude. Safavieh et al. prepared a paper-based microfluidic chip for HIV-1 detection employing printed graphene-modified silver electrodes. The LAMP product is captured by immobilizing the antibody on the paper surface. Impedance magnitude was measured after washing, and the impedance magnitude of

the chip containing the RT-LAMP products was significantly reduced [53].

Multiple Detection

From the perspective of monitoring emerging and re-emerging viruses for global health, detecting one virus at a time per sample may not be the best approach and it is worth discussing how to detect multiple viruses or multiple subtypes of the viruses in a single test with LAMP. For RNA viruses, due to their high mutation rate and diverse subtypes, multiple target sequence detection can largely relieve the pressure of testing different targets separately. However, the risk of primer dimer formation is aggravated by the fact that LAMP requires the involvement of multiple primers [54]. The design of multiple parallel channels and separate chambers on the microfluidic chip enables the simultaneous and independent detection of multiple target nucleic acids. Wang et al. performed multiplex detection of influenza virus using LAMP in the designed eight-channel microfluidic chip (Fig. 3a). Primer mixtures of seven pre-targeted sequences and negative controls were embedded in different microchambers. The template and the LAMP reaction mixture containing HNB are injected into the inlet and passed along the channel into eight different chambers. After incubation, the results of the different influenza A virus subtypes were recognized by color change [55]. A CD-like RT-LAMP microfluidic chip, using centrifugal force to distribute samples evenly into each reaction chamber, was designed to detect three swine enteric coronaviruses (Fig. 3b). The samples and reaction buffer are injected and sealed, followed by incubation, sample distribution, fluorescence signal acquisition, and data analysis in a supporting multifunctional device [56]. Even though these two microfluidic chips are capable of multi-gene detection, they rely on the off-chip sample pretreatment process and an additional custom-built kit, which limits their application for POC diagnostics.

Digital LAMP

Digital LAMP (dLAMP) enables absolute quantification of nucleic acid concentrations in a single test, reducing labor intensity and instrument complexity. dLAMP allows for high-throughput testing via physical compartmentalization, which helps to increase the number of people tested in a batch and improve the efficiency of the visit [60]. The reaction mixture is divided into small droplets, ensuring that each droplet contains one template molecule. After amplification, the number of droplets showing a positive signal is equal to the number of templates in the original sample, resulting in absolute quantification of the virus

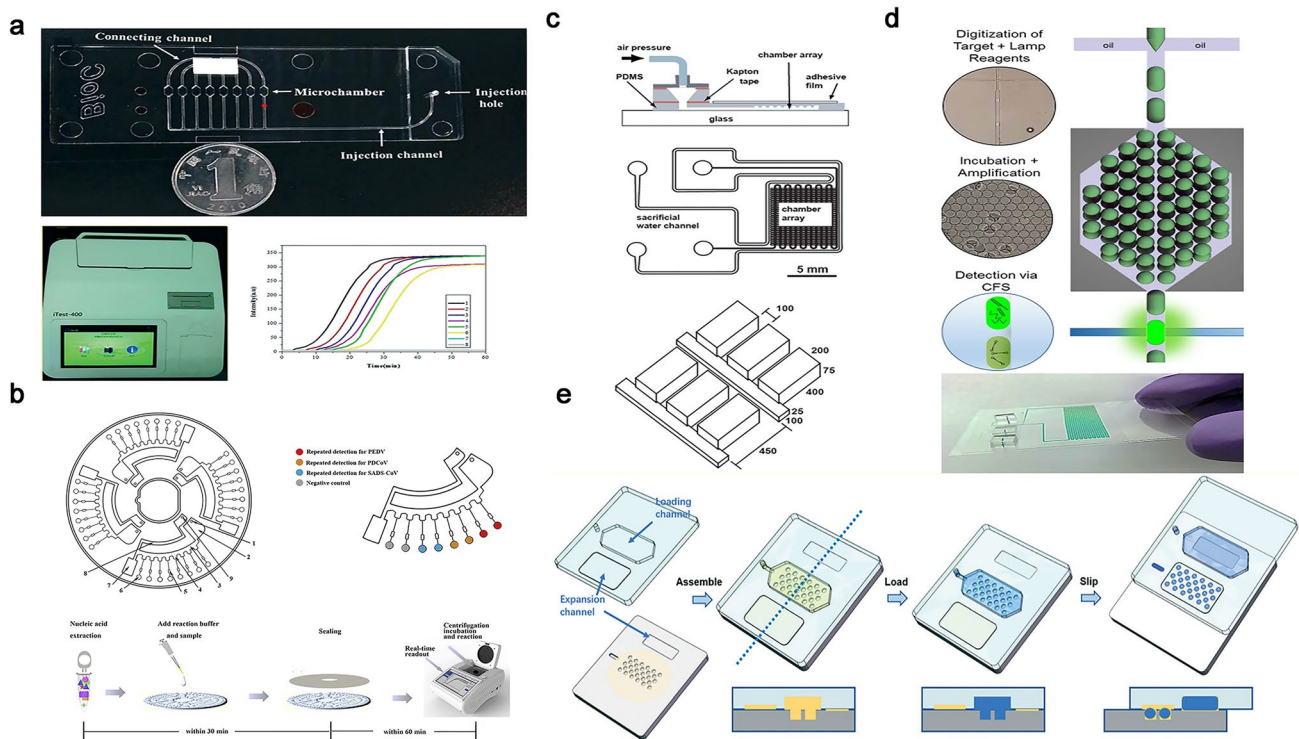


Fig. 3 Multi-detection microfluidic chip and digital LAMP microfluidic chip approaches. **a** An eight-channel microfluidic device for the detection of different influenza virus subtypes. Reproduced with permission from Wang et al. [55] (copyright 2018, Royal Society of Chemistry). **b** The CD-like microfluidic chip for three swine enteric coronaviruses detection. Reproduced with permission from Zhou et al. [56] (copyright 2020, Elsevier). **c** The self-digitization chamber-

based digital LAMP microfluidic chip. Reproduced with permission from Gansen et al. [57••] (copyright 2012, Royal Society of Chemistry). **d** Droplet microfluidic device for continuous flow digital LAMP analysis. Reproduced with permission from Rane et al. [58] (copyright 2015, Royal Society of Chemistry). **e** A droplet array SlipChip for SARS-CoV-2 detection. Reproduced with permission from Lyu et al. [59] (copyright 2021, Royal Society of Chemistry)

[61]. And then, digital LAMP-based microfluidic chips began to flourish. Based on the way the fluid is compartmentalized, it can be divided into two types of microfluidic chips, chamber based and droplet based [60]. The first self-digitization microfluidic chip-based digital LAMP was proposed by Gansen et al. in 2012 [57••] (Fig. 3c). The microfluidic array was embedded in the PDMS and sealed at the top, and sample loading with manual or automatic syringe pumps. A droplet-based continuous-flow digital LAMP microfluidic chip was proposed in 2015 (Fig. 3d). Using a classical cross between two microfluidic channels, the droplets were generated and then transferred to a heated region to perform amplification [58]. A droplet array SlipChip LAMP-based microfluidic device was developed by Lyu et al. for the detection of SARS-CoV-2 [59]. This slip chip partitions the liquid by the movement of its own slip, while this droplet formation in the organic phase effectively prevents potential cross-contamination and non-specific amplification (Fig. 3e).

The Combination of LAMP and Microfluidics

Integrated microfluidic devices reduce sample and reagent use since only small volumes of liquid are used. Furthermore, the entire assay process can be automated and integrated, greatly saving time and simplifying procedures [59, 62, 63]. It further makes point-of-care testing possible. The flow drive, flow control, sample preparation, amplicon detection, and result output procedures should be carefully designed to ensure the successful operation of LAMP on a microfluidic device, achieving the “sample to result” goal (Fig. 4) [64].

First, fluid actuation and control are necessary for LAMP miniaturization. Almost all microfluidic devices require power to drive the sample flow. An external pump unit is commonly used in most designs. For instance, syringe, peristaltic pumps, and piezoelectric pumps have been successfully used in microfluidics [64, 65]. However, an external apparatus adds additional costs to the

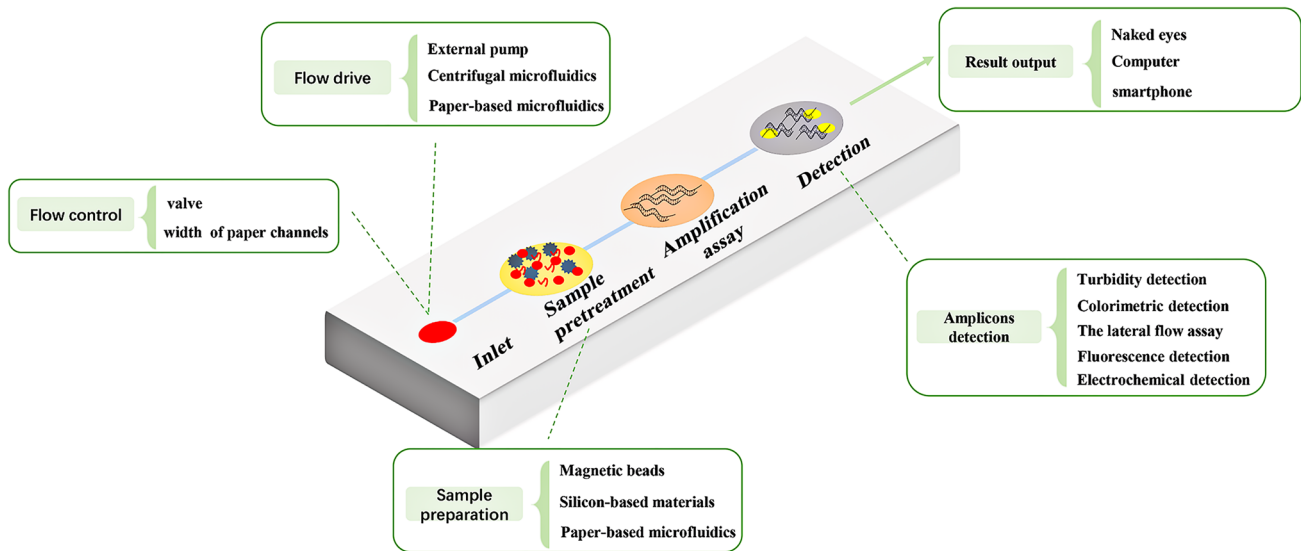


Fig. 4 The necessary steps for the combination of LAMP with microfluidics. An ideal microfluidic platform should consider fluid drive, fluid control, sample preparation, amplicon detection, and result output to achieve the “sample-result” goal

storage and transportation of the entire detection device. In recent years, pump-free microfluidic devices, such as centrifugal microfluidics and paper-based microfluidics, have been developed as an alternative. Centrifugal microfluidic devices drive the fluid via centrifugal force and

only require a motor. De Oliveira et al. also showed that a fidget spinner could generate sufficient centrifugal force. A valve is usually preset on the device for flow control by controlling the valve closure. In addition, paper-based microfluidics drive fluids via capillary action and do not

Table 1 Demonstration of different LAMP-based microfluidic devices for virus detection

Flow drive and control	Sample preparation	Detection method	Results output	Virus	LOD	Time (min)	Ref
Sample inlet by capillary action, and distribution of buffer by control of valve and centrifugal force	Off-chip	Lateral flow assay	Naked eye	H1N1	10 copies/reaction	55	[42]
Loading samples using capillary action of paper	Off-chip	Colorimetric detection with PH indicator	Smartphone	ZIKV	1 copy/μL	40	[77]
A slip-induced self-partitioning effect driven by surface tension	Off-chip	Colorimetric detection with calcein	Computer	HPV	Not mentioned	70	[32]
The liquid driven by centrifugal force and stopped by hydrophobic soft valves	Off-chip	Fluorescence detection with SYBR Green I	Computer	SARS-CoV-2	10 ⁻³ copies/reaction	10	[37•]
Centrifugation-based flow actuation	On-chip	Colorimetric detection with calcein	Computer	SARS-CoV-2	2 copies/reaction	70	[78]
The liquid driven by capillary force and stopped by hydrophobic soft valves	On-chip	Colorimetric detection with HNB	Naked eye	H1N1	87 copies/reaction	40	[79]
The liquid driven by capillary force and stopped by sealing pressure	On-chip	Fluorescence detection with SYBR Green I	Smartphone	SARS-CoV-2	100 copies/reaction	60	[80]

require any external equipment, controlling fluid movement based on channel width changes. The expansion of the channel size increases surface tension, reducing the fluid flow [37•].

Sample preparation via LAMP-based microfluidic devices is still challenging. Although microfluidic technology has made remarkable achievements in recent years, most microfluidic devices use off-chip nucleic acid extraction. This limits their use since additional manipulation outside the laboratory can cause sample contamination. Therefore, it is important to determine on-chip nucleic acid extraction techniques for ideal microfluidics. For instance, magnetic beads, silicon-based materials, and paper-based materials have been effective in nucleic acid extraction on chips [66, 67]. The combination and isolation of magnetic beads in nucleic acids can be controlled by adjusting the pH and salt concentration in the solution [68]. Modified silicon-based materials can be used for solid-phase extraction of nucleic acids on chips [68, 69]. The nucleic acid is physically or chemically electrostatically adsorbed on paper and is then eluted by a buffer solution [70, 71]. Lastly, it is important to determine methods for obtaining the assay results after on-chip LAMP. The amplicon detection methods have been described above. Visual inspection directly confirms the results using the naked eye. However, the accuracy of the results cannot be guaranteed since it is affected by subjective factors. Furthermore, naked eye testing has a high detection limit and can result in misdiagnosis. Therefore, electronic devices, especially smartphones, are essential for analyzing the results of a LAMP-based microfluidic platform. A smartphone camera could be used to capture the images on the chip before and after amplification. Specific software can then be used to analyze the image information to obtain quantifiable experimental results [72–74].

Conclusion

The recent advent of connected devices, such as smartphones in resource-limited settings where access to medical advice is limited due to inadequate health professionals, promotes communication with physicians without traveling long distances [75]. LAMP-based microfluidic devices can promote quick testing and timely communication with physicians when made widely available at the grassroots level. This would promote timely treatment and reduce the local healthcare burden. LAMP-based microfluidic devices for viral nucleic acid detection showing potential for deployment in the POC field and viral screening in large infectious disease outbreaks are shown in Table 1. Almost all LAMP-based microfluidic platforms can complete virus detection in about an hour with high sensitivity.

Large-scale deployment of nucleic acid testing and timely feedback of test results are critical to controlling the outbreak. The contamination-prone nature of LAMP leads to a high risk of false positives [76]. The combination of microfluidics with single-use and sealed microdevices will effectively remedy this dilemma. However, LAMP microfluidics also has some drawbacks that hinder its transformation from scientific research to practical application. To satisfy the conditions for nucleic acid detection outside the laboratory, the technical limitations imposed by on-chip LAMP must be considered very critically. From sample input to result output, where fluid control, nucleic acid extraction, target sequence amplification, amplicon detection, and result analysis all have an impact on the accuracy of the test. On top of that, a portable, rapid, accurate, specific, and sensitive nucleic acid detection technology is needed for outbreak response. The LAMP-based microfluidic platform is one of the preferred solutions for nucleic acid detection since it integrates multiple functions required for experiments on a single microchip, and its continuous development can promote the diagnosis of infectious diseases in the future.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no competing interests.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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