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REVIEW



## Loop-mediated isothermal amplification-based microfluidic chip for pathogen detection

Yuting Shang<sup>a</sup>, Jiadi Sun<sup>a</sup>, Yongli Ye<sup>a</sup>, Jumei Zhang<sup>b</sup>, Yinzhi Zhang<sup>a</sup>, and Xiulan Sun<sup>a</sup>

<sup>a</sup>State Key Laboratory of Food Science and Technology School of Food Science National Engineering Research Center for Functional Foods, Synergetic Innovation Center of Food Safety, Joint International Research Laboratory on Food Safety, Jiangnan University, Wuxi, Jiangsu, China; <sup>b</sup>Guangdong Institute of Microbiology, State Key Laboratory of Applied Microbiology Southern China Guangdong Provincial Key Laboratory of Microbiology Culture Collection and Application Guangdong Open Laboratory of Applied Microbiology, Guangzhou, China

### ABSTRACT

Due to the significant growth of food production, the potential likelihood of food contamination is increasing. Foodborne illness caused by bacterial pathogens has considerably increased over the past decades, while at the same time, the species of harmful microorganisms also varied. Conventional bacterial culturing methods have been unable to satisfy the growing requirement for food safety inspections and food quality assurance. Therefore, rapid and simple detection methods are urgently needed. The loop-mediated isothermal amplification (LAMP) technology is a highly promising approach for the rapid and sensitive detection of pathogens, which allows nucleic acid amplification under isothermal conditions. The integration of the LAMP assay onto a microfluidic chip is highly compatible with point-of-care or resource-limited settings, as it offers the capability to perform experiments in combination with high screening efficiency. Here, we provide an overview of recent advances in LAMP-based microfluidic chip technology for detecting pathogens, based on real-time or endpoint determination mechanisms. We also discuss the promoting aspects of using the LAMP technique in a microfluidic platform, to supply a guideline for further molecular diagnosis and genetic analysis.

### KEYWORDS

Loop-mediated isothermal amplification; microfluidic chip; pathogens

## Introduction

Food safety is a scientific discipline that describes the preparation, treatment, and storage of food products in ways that enable prevention of foodborne diseases. Currently, this discipline has gained worldwide attention. Among food contaminants, foodborne pathogens are a serious threat to public health and the global economy. To address this global challenge and provide safe food for consumers, numerous rapid methods have been proposed during the past decade, such as nucleic acid-based (e.g., PCR, NASBA, and RPA), immunological-based (e.g., ELISA, LFD, and LAT), and bio-sensor-based (e.g., optical, electrochemical, and mass-based biosensors) methods (Zhao et al. 2014). Nevertheless, two of the most challenging issues still remain unsolved: (1) the limitations of sample preparation from food matrix and (2) the insufficient sensitivity of detection methods. Therefore, simple, accurate, sensitive, high-throughput, and easy-to-use methods are required for the on-demand detection of pathogens in food samples.

The loop-mediated isothermal amplification (LAMP) technology is an emerging isothermal nucleic acid amplification method developed by Notomi et al. in 2000 (Notomi et al. 2000). The basic principle of LAMP is auto-cycling strand displacement DNA synthesis in the presence of Bst DNA polymerase. The reaction results in  $10^6$ – $10^9$  copies of

target DNA within 30–60 min under isothermal conditions between 60 and 65 °C. The LAMP reaction requires four primers to recognize six distinct regions of the target: a forward inner primer (FIP), a backward inner primer (BIP), a forward outer primer (F3), and a backward outer primer (B3). The amplification process can be divided into two phases. At the initial phase, a dumbbell structure is formed based on the Bst DNA polymerase with high strand displacement activity and specifically designed primers. This dumbbell structure is a basic template for the second phase reaction. The second phase is the cyclic amplification and only two inner primers are needed for this phase. The final products of the LAMP reaction are mixtures of stem-loop DNAs. These stem-loop DNAs contain several repeats of target and cauliflower-like multiple loop structures. Prior to the amplification step, additional reverse transcriptase enzyme is required to transcript RNA into cDNA for RNA detection (Li et al. 2017). Furthermore, the detection efficiency has been improved by adding an additional LAMP loop primer (LF/LB), thus shortening the reaction time to be within 30 min (Nagamine, Hase, and Notomi 2002). Since LAMP introduction as a new detection technology, it has been widely used in medicine, biology, environmental monitoring, and food industries. The advantages of LAMP compared to traditional PCR are listed in Table 1.

**Table 1.** The advantages of LAMP compared with PCR.

	LAMP	PCR
Instrumental requirements	Thermostat water bath or thermos flask, low cost	PCR instrument, high cost
Speed	Quickly, 30–60 min	Slowly, 2–3 h
Specificity	Highly, 4–6 primers correspond to 6–8 target sites	Lowly, 2 primers correspond to 2 target sites
Sensitivity	10 <sup>0</sup> copies, lower 10 times than PCR	10 <sup>1</sup> –10 <sup>2</sup> copies
Amplification efficiency	Highly, 10 <sup>9</sup> fold in 30–60 min	Lowly, 2 <sup>30</sup> fold in 2–3 h
Tolerance	Simple sample preparation, the demand for nucleic acids quality is low, and the impurity tolerance is high, such as MEM medium, serum, plasma, urea	The demand for nucleic acids quality is high, and the impurity tolerance is low
Judgment of result	Easy to distinguish, various methods, such as electrophoresis, turbidity, fluorescence probe	High equipment requirements, such as electrophoresis and gel imaging system

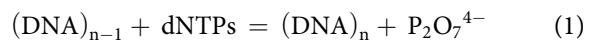
In addition to LAMP, other isothermal amplification techniques have also been invented, including nucleic acid sequence-based amplification (NASBA), rolling circle amplification (RCA), strand displacement amplification (SDA), helicase dependent amplification (HDA), and single primer isothermal amplification (SPIA) (Zhao et al. 2015). While these methods have their potential advantages respectively, there still exist shortcomings to overcome, such as nonspecific amplifications cause high background noise, complex primers complicate the assay process, and low tolerances need optimal reaction system. In contrast, LAMP has been shown to be faster and more stable, sensitive, and specific for nucleic acid detection. It is highly specific for the target sequence, because the binding sites are recognized by using four to six primers. It also has the ability to amplify medium- to long- range of nucleic acids, which makes it suitable for detection of various DNA or RNA sequences. What's more, the assay is stable against some amplification inhibitors and its detection limit is 10 copy or even more less template (Wong et al. 2018). As LAMP advances, it can be integrated into a microchip to ease transportation to the site of diagnosis. LAMP methods and their applications have been summarized previously (Li et al. 2017; Kokkinos et al. 2014); however, the recent development of LAMP methods on microfluidic chip has not been specifically reviewed. Therefore, this review focuses on elucidating the improvement of LAMP technology for microfluidic chips. Microfluidic chips or lab-on-a-chips originated in the late 1970s (Terry, Jerman, and Angell 1979); and the realization of integrated microfluidic systems has rapidly led to improvements in miniaturization. The microfluidic chip possesses unique advantages: (1) preventing contamination, (2) reducing cost, (3) running several reactions in parallel and, (4) simplifying the amplification reactions (by eliminating the need for laboratory equipment) (Daw and Finkelstein 2006). This paper reviews the recent development of LAMP methods performed on microfluidic chip for the detection of pathogens over the last 5–8 years. The LAMP amplicon detection can be divided into two categories: monitoring at the end-point and real-time monitoring of the LAMP reaction. According to different detection methods, turbidity, dyes, electrochemistry, electrophoresis, and immunoassay modalities are leveraged for end-point detection methods. Turbid, fluorescence, electrochemical, and the other mechanisms are used for real-time detection methods (Fig. 1). Each method is supported with examples for better

understanding, and the further considerations of the design characteristics of LAMP-based microfluidic chip are discussed.

## End-point detection methods

### Turbidity detection

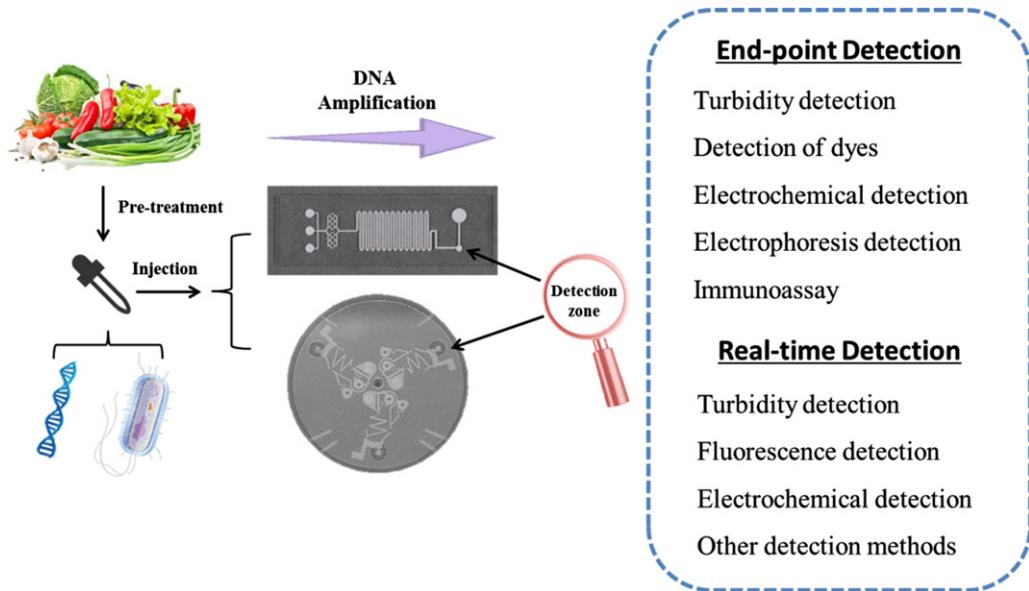
In the LAMP reaction process, a large number of pyrophosphate ions are released from deoxyribonucleotide triphosphates (dNTPs) (Equation 1). Then, the ions react with magnesium ions to yield a white magnesium pyrophosphate precipitate ( $Mg_2P_2O_7$ ) (Equation 2). The byproduct  $Mg_2P_2O_7$  increases the turbidity of the background solution, which can be visualized with the naked eye either with or without the help of centrifugation (Zhang, Lowe, and Gooding 2014).



There is no extra instrumental cost in monitoring LAMP by using this method but its sensitivity is lower and misjudgment easily happens, particularly when the amplification product is faint and the white precipitate is not obvious. Therefore, the introduction of optical instruments into detection systems, including turbidimeters, optical fibers and spectrophotometers, can improve the sensitivity and realize real-time detection. For example, Fang et al. (2010) designed eight parallel reaction channels for in-chip LAMP reaction. Each channel only requires 0.4  $\mu$ L sample volume and the reaction completes within 1 h. With the integration of optical fibers, this can realize the real-time monitoring of LAMP process and its quantitative analysis. The other examples and details will be discussed in section 3.1.

### Detection of dyes

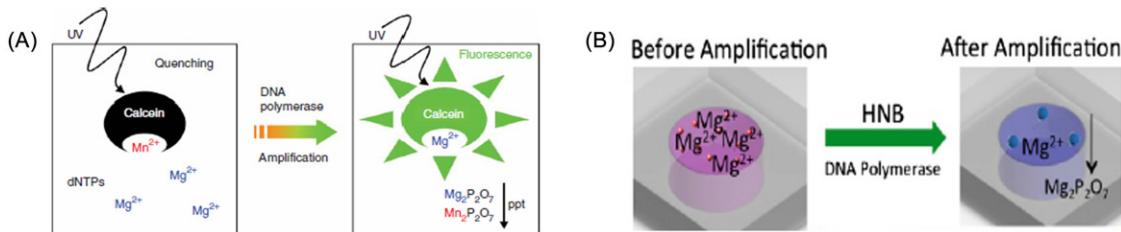
The sensitivity of the detection using dyes (Table 2) is considerably higher than that obtained via turbidity alone. Metal ion indicators reflect the change of  $Mg^{2+}$  concentration in the reaction system, such as hydroxynaphthol blue (HNB), eriochrome black T (EBT), and calcein. DNA-binding dyes selectively bind to double-stranded DNA (dsDNA). The formation of the dye-dsDNA complex causes a visible color change of the dye, such as ethidium bromide (EB), propidium iodide (PI), SYBR Green I, and SYTO-81. The



**Figure 1.** Schematic of the workflow of a LAMP-based microfluidic chip for highly throughput screening and detection of target bacterial pathogen. There are mainly four steps: sample pretreatment, sample injection, DNA amplification, and detection. The detection methods can be divided into two categories: monitoring at the end-point and real-time monitoring; furthermore, each category includes different methods.

**Table 2.** Various dyes used for LAMP detection.

Dye	Mechanism	Color before and after amplification	Inhibit LAMP
Calcein	Metal ion indicators, fluorescence enhancement with the displacement of $Mn^{2+}$ from calcein to $P_2O_7^{4-}$	Orange /Green	No, but additional added $Mn^{2+}$ inhibit reaction
Hydroxynaphthol blue (HNB)	Metal ion indicators, color changes from purple to blue with the reduction of $Mg^{2+}$ that can be observed by naked eye	Purple/Blue	No
Eriochrome black T (EBT)	Metal ion indicators, color changes from red to blue with the reduction of $Mg^{2+}$ that can be observed by naked eye	Red/Blue	No
Ethidium bromide (EB)	DNA-binding dyes, intercalated with dsDNA	No color/Orange	No
Propidium iodide (PI)	DNA-binding dyes, intercalated with dsDNA	No color/Red	Yes
SYBR Green I	DNA-binding dyes, intercalated with dsDNA	Dark orange/Green	Yes
SYTO-81	DNA-binding dyes, intercalated with dsDNA	Orange/Green	No
PicoGreen	DNA-binding dyes, intercalated with dsDNA	Light orange/Green	Yes
EvaGreen	DNA-binding dyes, intercalated with dsDNA	Orange/Green	No
Gene Finder	DNA-binding dyes, intercalated with dsDNA	Orange/Green	Yes



**Figure 2.** Principles of the detection using metal ion indicators. (A) Calcein. (B) HNB. Reprinted with permission from Refs. Kanda et al. (2008) and Goto et al. (2009), respectively.

dyes do not inhibit the amplification reaction and the color change can be easily distinguished under natural light and UV light. Both calcein and HNB are common dyes that do not inhibit the LAMP reaction. As shown in the schematic

drawing (Fig. 2A), calcein initially combines with  $Mn^{2+}$ , thus quenching calcein fluorescence and the color of the LAMP reaction solution appears orange. As the amplification proceeds,  $Mn^{2+}$  is displaced by calcein to newly

**Table 3.** Comparison of LAMP-based microfluidic chips using dyes detection.

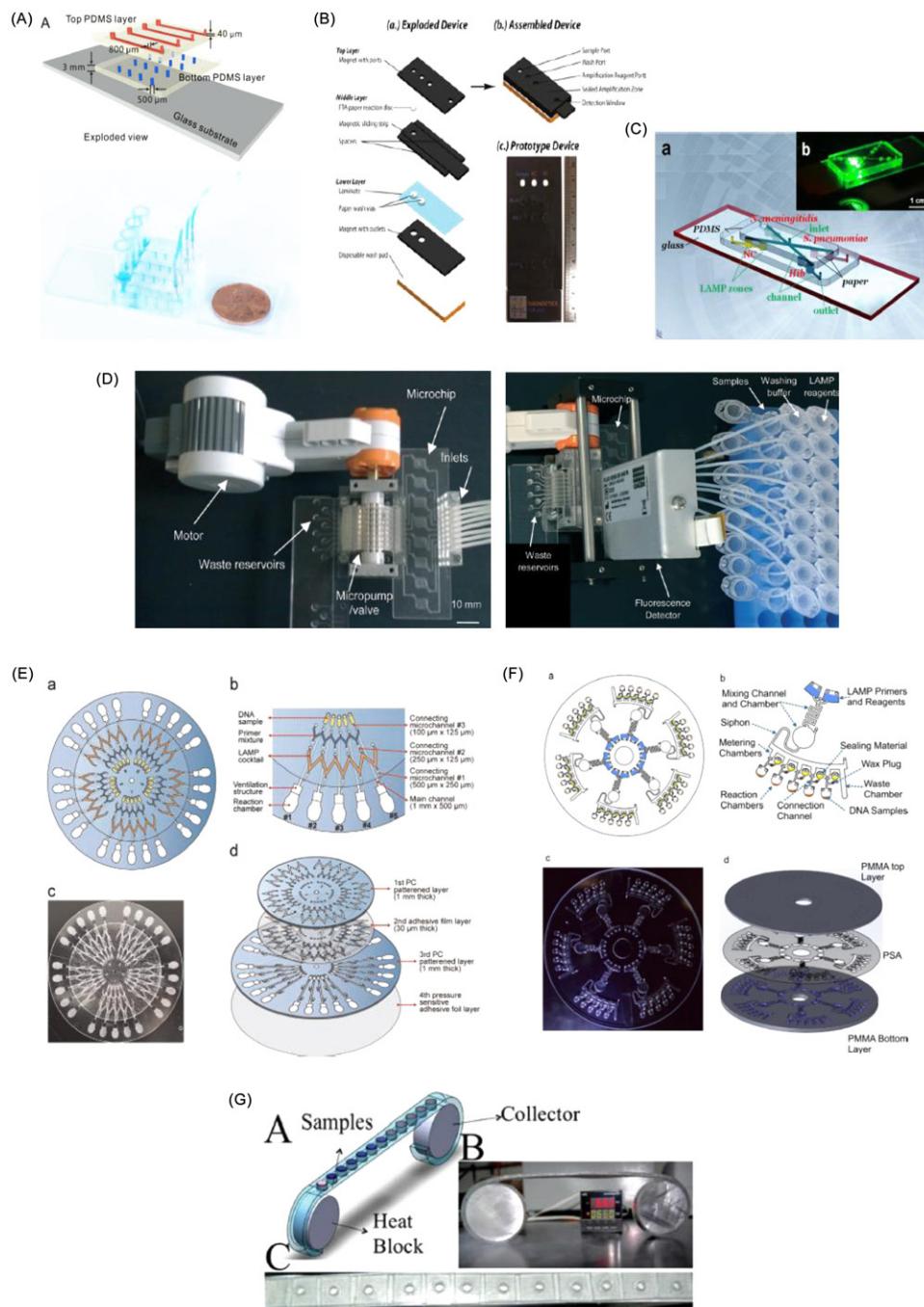
Pathogenic bacteria	Dye	Material and shape of chip	Detection time	Detection limit	Quantitative/qualitative	Degree of automation <sup>a</sup>	References
<i>Escherichia coli</i>	Calcein	PDMS/Rectangle	70 min	3 copies/µL	Qualitative	Medium	(Chen et al. 2017)
<i>Escherichia coli</i>	Calcein	PDMS/Rectangle	55 min	270 copies/µL	Qualitative	High	(Fang et al. 2012)
<i>Escherichia coli</i>	HNB	Paper	60 min	$4.14 \times 10^3$ copies/µL	Semi-quantitative	Low	(Hongwarittorn, Chaichanawongsaroj, and Laiwattanapaisal 2017)
<i>Escherichia coli</i>	SYBR Green I	Paper	60 min	5 copies/reaction	Qualitative	Low	(Connelly, Rolland, and Whitesides 2015)
<i>Escherichia coli</i>	SYTO-9	PDMS/Rectangle	60 min	10 copies/reaction	Quantitative	High	(Liu, Mauk, and Bau 2011)
<i>Escherichia coli</i>	[Ru(phen) <sub>2</sub> dppz] <sup>2+</sup>	Paper	30 min	100 copies/reaction	Quantitative	Medium	(Li et al. 2018)
<i>E. coli</i> O157:H7	EBT	PMMA/Circle	60 min	40 copies/µL	Qualitative	Medium	(Seo et al. 2017)
<i>E. coli</i> O157:H7	EBT	PC/Circle	60 min	15.2 copies/µL	Qualitative	Medium	(Oh et al. 2016)
<i>E. coli</i> O157:H7	EvaGreen	Silicon/Rectangle	/	3 CFU/reaction	Quantitative	Medium	(Duarte et al. 2013)
<i>Salmonella</i>	Calcein	PMMA/Circle	60 min	$3 \times 10^{-5}$ ng/µL	Qualitative	Medium	(Sayad et al. 2018)
<i>Salmonella</i>	HNB	CD、DVD、BD/Circle	45 min	65 fg/µL	Quantitative	Medium	(Santiago-Felipe et al. 2016)
<i>Salmonella</i>	SYBR Green I	PMMA/Circle	70 min	$5 \times 10^{-3}$ ng/µL	Quantitative	Medium	(Sayad et al. 2016)
<i>Salmonella</i>	SYBR Green I	PMMA/Circle	62 min	$2.5 \times 10^{-3}$ ng/µL	Qualitative	Medium	(Uddin et al. 2015)
<i>Salmonella</i>	SYTO-62	COC/Rectangle	40 min	10 copies/µL	Quantitative	High	(Sun et al. 2015)
<i>Salmonella</i>	Fisetin	Paper	70 min	$1.7 \times 10^2$ CFU/mL	Semi-quantitative	Low	(Trinh and Lee 2018)
<i>Vibrio parahaemolyticus</i>	Calcein	PDMS/Circle	60 min	7.2 copies/µL	Qualitative	Medium	(Xia et al. 2016)
<i>Vibrio parahaemolyticus</i>	EvaGreen	PMMA/Circle	30 min	0.28 pg/µL	Quantitative	Medium	(Zhou et al. 2014)
<i>Staphylococcus aureus</i>	EBT	PMMA and paper	30 min	1 fg/µL	Semi-quantitative	Low	(Kim et al. 2018)
<i>Staphylococcus aureus</i>	Calcein	PE/Ribbon	60 min	200 CFU/mL	Qualitative	Medium	(Safavieh, Ahmed, Sokullu, et al. 2014)
<i>Staphylococcus aureus</i>	Calcein	PDMS/Pentagon	90 min	24 CFU/reaction	Qualitative	Medium	(Jiang et al. 2016)
<i>Staphylococcus aureus</i>	EvaGreen	PC/Circle	45 min	10 copies/1.45 µL	Quantitative	Medium	(Huang et al. 2017)
<i>Neisseria meningitidis</i>	Calcein	PDMS and paper/Rectangle	45/60 min	3 copies/reaction	Quantitative	Medium	(Dou et al. 2014; Dou et al. 2017)
<i>Campylobacter jejuni</i>	SYTO-82	COP/Rectangle	45 min	10 copies/µL	Quantitative	Medium	(Tourlousse et al. 2012)
<i>Streptococcus agalactiae</i>	EvaGreen	PDMS/Rectangle	65 min	20 copies/µL	Quantitative	High	(Chang Yang, Wang, et al. 2013)
<i>Bacillus subtilis</i>	Crystal violet	Paper	60 min	$2.2 \times 10^3$ copies/µL	Quantitative	Medium	(Roy et al. 2017)

<sup>a</sup>The degree of automation is graded based on the number of the analytical steps within a microchip. A fully automation device should be integrated with sample processing, amplification, and detection units to realize on-site pathogens detection. Medium-level automation needs less manual operations than low-level automation, and high-level automation further includes upstream sample processing step.

generated  $\text{P}_2\text{O}_7^{4-}$ , which results in the recovery of the green fluorescence. Additionally, the free calcein combines with residual  $\text{Mg}^{2+}$ , thus enhancing the fluorescence signal (Kanda et al. 2008). HNB shows a similar mode of action where the color of the reaction system changes from purple to blue due to the decrease of  $\text{Mg}^{2+}$  concentration in the solution (Fig. 2B) (Goto et al. 2009).

Table 3 lists various LAMP-based microfluidic chips that utilized the dye detection method for pathogen detection in recent years. As shown in Table 3, polymers and paper are the most commonly used materials for microfluidic device fabrication. Chen et al. (2017) developed a PDMS microchip, in which each reaction chamber contains low-melting-point agarose and all LAMP reagents except the DNA samples. This microchip can simultaneously detect four pathogens. Calcein was used to detect the amplicons with a limit of detection (LOD) of 3 copies/µL for *Escherichia coli* (Fig. 3A). Paper substrate is inexpensive and has a high surface-

to-volume ratio and the paper-based microfluidic devices have emerged as a low-cost and easy-to-use platform. Connelly, Rolland, and Whitesides (2015) developed a cellulose paper microchip that integrates sample preparation and LAMP amplification with end-point detection by using SYBR Green I dye and the LOD for *E. coli* was 5 cells/reaction. Cellulose paper is a suitable matrix for DNA adsorption and isolation (Fig. 3B). The paper that was immobilized by polyethylenimine (PEI) achieved semi-quantification of the initial concentration of LAMP-generated DNA as low as  $4.14 \times 10^3$  copies/µL. The PEI reacted with the free HNB in LAMP system, producing a blue color deposit on the paper device, the distance of which correlated with the amount of DNA in the sample (Hongwarittorn, Chaichanawongsaroj, and Laiwattanapaisal 2017). Furthermore, a hybridized PDMS/paper microfluidic chip was developed for rapid and sensitive detection of *N. meningitidis* using calcein and the LOD was three copies per reaction (Dou et al. 2014).



**Figure 3.** Schematic of LAMP-based microfluidic chips using dyes detection. (A) PDMS-based microchip for pathogen detection in gel-LAMP reaction. (B) Paper-based microchip for LAMP amplification and detection. (C) PDMS/paper hybrid microchip for multiplexed bacterial meningitis diagnosis. (D) Integration of microchip with the Mainstream micropump for *Salmonella* spp. detection. (E) Centrifugal LAMP microchip with zigzag-shaped dispensing microchannels. (F) Centrifugal LAMP microchip with square-wave microchannels. (G) LAMP-based cassette device. Reprinted with permission from Refs. Chen et al. (2017), Connelly, Rolland, and Whitesides (2015), Dou et al. (2017), Sun et al. (2015), Oh et al. (2016), Sayad et al. (2018), and Safavieh, Ahmed, Sokullu, et al. (2014), respectively.

Through further design, three types of bacterial meningitis could be detected simultaneously with this hybridized system (Fig. 3C) (Dou et al. 2017).

As shown in Table 3, the shape of major microfluidic chips is rectangular or circular. The rectangular microfluidic chip needs extra pumping and valve systems. For example, an eight-chamber microfluidic chip was connected to an eight-channel peristaltic micropump with integrated magnetic bead-based sample preparation and LAMP reagents for the detection of *Salmonella* spp. in food samples. The LOD of the system was 10 copies/µL using SYTO-62 dye (Fig.

3D) (Sun et al. 2015). However, the centrifugal microfluidic device dispenses LAMP reagents without the need for micropumps or microvalves thus allowing the multiplexed detection of pathogens. Oh et al. (2016) designed a centrifugal LAMP-based microfluidic chip which LAMP cocktail, primer mixtures, and DNA sample solutions that were sequentially and accurately metered to the reaction chambers by zigzag-shaped microchannels and RPM control. Twenty-five samples could be analyzed within 60 min and the LOD of *E. coli* O157:H7 was 15.2 copies/µL using EBT dye (Fig. 3E). In a further study, LAMP reagents were

**Table 4.** Various electroactive reagents for LAMP detection.

Electroactive reagent	Electrochemical technique	Electrode	References
Hoechst 33258	LSV	Carbon	(Safavieh et al. 2012)
Methylene blue (MB)	SWV	ITO	(Luo et al. 2014)
Methylene blue (MB)	DPV	Gold	(Hsieh et al. 2012)
Os[(bpy) <sub>2</sub> DPPZ](PF <sub>6</sub> ) <sub>2</sub>	SWV	Screen-printed electrode	(Safavieh Ahmed, Ng, et al. 2014)
Ru(Phen) <sub>3</sub> <sup>2+</sup>	CV	Gold	(Ahmed et al. 2013)
2'-deoxyguanosine 5'-triphosphate (dGTP)	DPV	Carbon	(Zhang et al. 2011)

loaded into the mixing chamber through a zigzag channel and flowed to the metering chambers, where they were finally transferred to the amplification chambers, which have been injected into DNA samples. The sealing chambers were pre-loaded with UV curable adhesives and the process was controlled using a wax valve. The LOD for *Salmonella* was  $5 \times 10^{-3}$  ng/ $\mu$ L via visual observation of the color change of SYBR Green I dye (Sayad et al. 2016). With further improvement, this could detect more samples at the same time and skip the dye-adding step, because the SYBR Green I dye was replaced by calcein, which can be added to pre-amplification samples without inhibiting the LAMP reaction (Fig. 3F) (Sayad et al. 2018). Safavieh, Ahmed, Sokullu, et al. (2014) developed a novel microfluidic chip with a flexible ribbon polyethylene substrate material. The device is capable of detecting 30 CFU/mL of *E. coli* and 200 CFU/mL *Staphylococcus aureus* within 60 min using HNB and calcein dyes, respectively (Fig. 3G). The detection time of microfluidic chips mostly ranges 45–60 min, while the detection limit differs between each of these.

Due to the strong background fluorescence is induced by nonspecific dsDNA, the common used single dye is hard to clearly identify the weak-positive reaction. Ding et al. (2015) reported a mixed-dye containing HNB and SYBR Green I, which could improve the detection sensitivity and avoid the empirical preset of cutoff intensity values, making the result more accurate. Shortly afterwards, Pang et al. (2017) found that this mixed-dye is very suitable to be applied on microchips, so a self-priming compartmentalization (SPC) microchip was developed to detect *Vibrio parahemolyticus* by applying a mixed-dye-loaded LAMP reaction. They further combined this method with a self-priming polydimethylsiloxane(PDMS)/paper hybrid microchip to improve LAMP reaction performance and realized multiplex foodborne pathogens detection (Pang et al. 2018). Although dye detection is less expensive than other methods (it does not require readers or signal processing), its main drawback is that it is mostly qualitative, and much room remains to be improved.

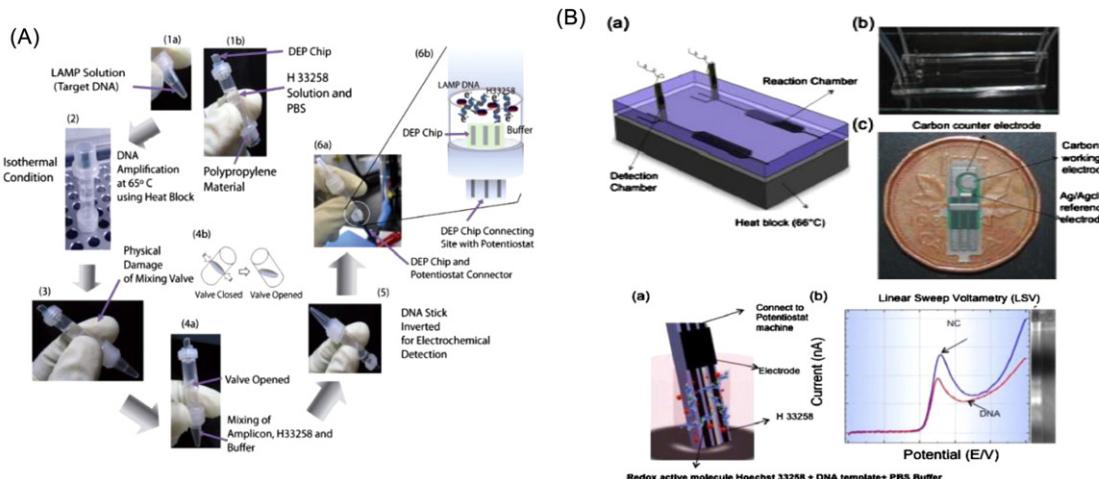
### Electrochemical detection

Electrochemical sensing has shown promising advantages, such as rapid speed, high sensitivity, low cost, and more readily application in point-of-care testing (POCT) platforms. The combination of it with LAMP reaction can detect various analytes. The electrochemical detection is carried out by employing probe-immobilized electrode modified with a redox reporter or based on the measurement of the interactions occurring between electroactive molecules

and the DNA products or byproducts (e.g. PPi (Xie, Tang, and Tang 2018) or H<sup>+</sup> (Zhao et al. 2018)), which are generated during the amplification process (Qi et al. 2018). The LAMP reactions can be detected after the amplification reactions or be monitored in real time. The electrochemical signals can be recorded by linear sweep voltammetry (LSV), square wave voltammetry (SWV), differential pulse voltammetry (DPV), or cyclic voltammetry (CV) (Table 4). In this section, we will focus on the electrochemical sensing chip for monitoring the LAMP reactions at the endpoint.

Electrochemical biosensor is an end-point method that incorporates biological recognition into the detection system. Several electrochemical biosensor-based methods have been reported for monitoring the LAMP reaction. Sun et al. (2010) developed an electrochemical DNA sensor to detect the *Yersinia enterocolitica* gene sequence, immobilizing specific ssDNA probe onto a carbon ionic liquid electrode (CILE). An MB indicator as an electrochemical redox molecule was used to label the LAMP amplicons. The assay achieved a dynamic concentration range from  $1.0 \times 10^{-11}$  to  $1.0 \times 10^{-6}$  mol/L and the LOD was  $1.76 \times 10^{-12}$  mol/L by using DPV method. In another study, Nakamura et al. (2010) combined the LAMP reaction with an electrochemical DNA biochip to detect the CYP2D6 gene copies. Generally, the electrochemical biosensor is used to detect amplicons after LAMP reaction. The specificity of it is high as the directly sequence-specific DNA hybridization. However, it is costly and time-consuming to prepare this biosensor and the dynamic concentration range is narrow.

In another strategy, some immobilization-free LAMP methods have been developed. The binding of a redox molecule with LAMP amplicons can cause a change in the measured values. For instance, Ahmed et al. (2009) integrated the LAMP reaction with a disposable electrochemical printed (DEP) chip in a stick-like incubator for the detection of maize CBH 351. The device consisted of an amplification layer and a detection layer that have been separated by a mixing valve, and a DEP chip was attached to its end. The amplification layer was where the LAMP reaction occurred. The detection layer was where Hoechst 33258 resided to mix with amplicons. The mixing valve was opened after amplification so that the interaction phenomenon of the DNA-Hoechst 33258 can be measured with the DEP chip using the LSV method. The sensitivity of the assay was 300 CFU/reaction during 20 min (Fig. 4A). Another electrochemical LAMP-based microfluidic chip using the Hoechst 33258 indicator to detect bacteria was also reported. The device was composed of two parallel channels to detect the negative control and DNA sample. Each channel contained a reaction chamber, an active valve, and an electrochemical



**Figure 4.** Schematic of LAMP-based microfluidic chips using electrochemical detection. (A) Stick-like microchip for electrochemical detection of maize CBH 351 GMO. (B) Microfluidic electrochemical assay for *E. coli* detection. Reprinted with permission from Refs. Ahmed et al. (2009) and Safavieh et al. (2012), respectively.

detection chamber. The detection of bacteria was implemented electrochemically using the LSV method since the dsDNA binding with Hoechst 33258 causes a significant decrease in the current response. The sensitivity of the assay was 48 CFU/mL during 35 min (Fig. 4B) (Safavieh et al. 2012). All the methods described above could yield a valuable yes/no answer or semi-quantitative results, but do not possess fully quantitative capabilities. This is because the final concentration of amplifications generally does not correlate well with the initial copy number present in a sample, thus real-time electrochemical detection is needed, and we will describe in Section 3.2.

### Electrophoresis detection

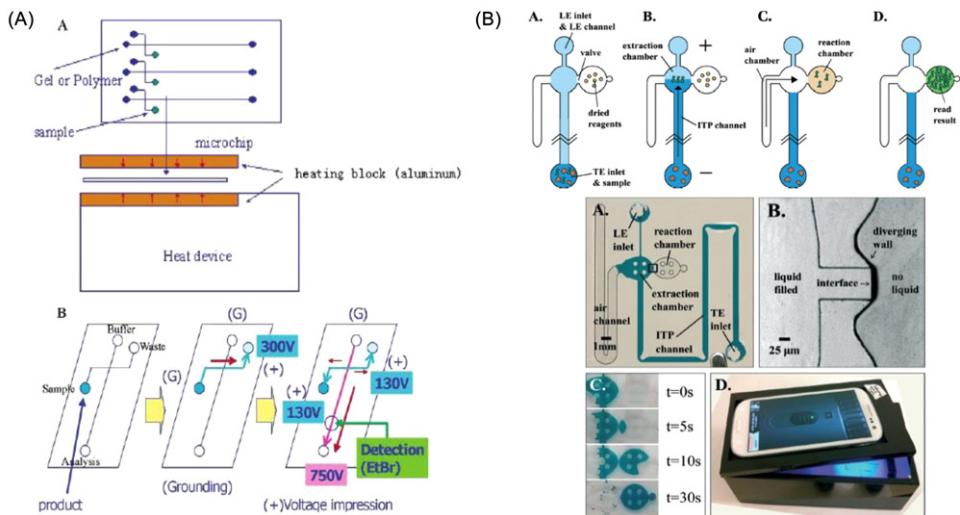
Electrophoresis is a method for the separation of macromolecules (DNA, RNA, and proteins) based on their size and charge. The products of LAMP can be directly detected via gel electrophoresis (GE) due to their different amplicon lengths. After staining with fluorescence dyes (commonly ethidium bromide and SYBR Green dye), the gel will show a reproducible ladder-like banding under UV gel imaging. Compared to indirect detection methods, i.e. turbidity and colorimetry, the recognition of a specific length of an amplicon can reduce the risk of nonspecific detection. However, GE is usually used as a post-processing method for LAMP detection and has not been used in microfluidic chips.

Capillary electrophoresis (CE) offers the advantage of on-chip multiplex detection and the measurements of mobility are relatively simple (e.g. using a single, homogeneous buffer chemistry). Hataoka et al. (2004) have been the first to combine LAMP with electrophoresis in a simple cross-channel PMMA microchip. This chip can detect prostate specific antigen cDNA with an LOD of 23 fg/μL within 15 min (Fig. 5A). Moreover, a novel polyacrylamide (PAA) gel-based microchamber was developed for single molecule detection. Due to the porosity of PAA, ions could diffuse into its gel matrix to initiate amplification, and electric signals could penetrate the gel structure to manipulate the motion of encapsulated molecules (Lam et al. 2008).

Isotachophoresis (ITP) is also an electrophoresis technique to separate and concentrate ionic molecules that only requires leading electrolyte (LE) buffer, trailing electrolyte (TE) buffer and an electric field. It can be used to simultaneously identify multiple samples, which allows for low analytic concentrations. Borysiak, Kimura, and Posner (2015) utilized ITP and LAMP to extract and amplify nucleic acids from complex matrices in less than one hour. The analytic process includes five steps: (1) adding sample to the TE buffer, (2) adding LE and TE sample mixtures to the corresponding channel, (3) applying an electric field for ITP, (4) heating the chip, and (5) taking an image of the chip with a mobile phone. This device has an LOD of  $10^3$  CFU/mL for the detection of *E. coli* O157:H7 from milk samples, which is two orders of magnitude lower than LAMP in unprocessed samples (Fig. 5B). In general, reports about the detection of traditional PCR using electrophoresis-based microfluidic chips are more numerous than those for LAMP reaction. However, LAMP is more suitable for integration with a chip and the research of this aspect should be further advanced.

### Immunoassay

Immunoassay, including lateral flow dipstick (LFD) and enzyme-linked immunosorbent assay (ELISA), has been used for the detection of the LAMP reaction. On the LFD strip, the test line captures biotin-labeled LAMP amplicons that have been hybridized with FITC-labeled DNA probes, and gold-based anti-FITC antibodies are introduced to develop a readable output. Non-hybridized FITC probes bind to gold-labeled anti-FITC antibody antibodies, thus forming a double complex without biotin that moves through the test line to the control line. Although this is the original strategy that combines LAMP with LFD (Ge et al. 2013), there still exists vast improvement space to develop in this field. For example, using different fluorophore-labeled primers to allow simultaneous detection of multiple results (Foo et al. 2017), using enzyme- or nanomaterials-based enhancement strategies to improve detection



**Figure 5.** Schematic of LAMP-based microfluidic chips using electrophoresis detection. (A) Integration of LAMP reaction and electrophoresis-based analysis on one chip. (B) Integration of isotachophoresis separation and LAMP reaction on one chip. Reprinted with permission from Refs. Hataoka et al. (2004) and Borysiak, Kimura, and Posner (2015), respectively.

sensitivity (Cheng et al. 2017), and even elaborately coupling target-specific strand exchange reactions to demonstrate specific detection (Phillips et al. 2018). However, the nucleic acid amplification process and LFD are still separately performed, which entails multiple processing steps and increases the risks of cross-contamination. Therefore, there is a strong demand to develop a new integrated LFD that can achieve on-site naked-eye detection.

Rodriguez et al. (2016) designed a paper-fluidic chip integrating nucleic acid extraction, LAMP, and LFD for the detection of human papillomavirus (HPV) 16 DNA directly from crude cervical specimens in less than 60 min. In the LAMP process, the loop primers (LF and LB) were tagged with FITC and biotin, respectively. The test strip consists of streptavidin-conjugated gold nanoparticles, an anti-FITC test line, and a biotin control line, and enables immediate visual readout (Fig. 6A). The LFD method has particular advantages in specificity since the probe targets a specific complementary sequence within the LAMP product as opposed to nonspecific binding of dsDNA by intercalating dyes. Similarly, Choi et al. (2016) developed an integrated paper-based device that incorporates LAMP and LFD, and achieved a detection limit of as low as  $3 \times 10^3$  copies of target DNA (Fig. 6B). Moreover, to eliminate the high risk of carry-over contamination, Jung et al. (2015) designed a novel centrifugal microfluidic chip that integrates RT-LAMP and three separate LFDs on a compact disk to detect the H1N1 virus (Fig. 6C). Lee et al. (2016) utilized a slidable plate to manipulate the fluidic control on a chip and sequentially to perform the operations of the LAMP reaction and LFD detection (Fig. 6D). Park et al. (2017) further integrated glass-microbead-based DNA extraction into a rotary microfluidic system to simplify the sample pretreatment process, and introduced two fluorescence-labeled primer probes into LAMP reaction to simultaneously detect *Salmonella typhimurium* and *Vibrio parahaemolyticus* with a limit of detection of 50 CFU in 80 min (Fig. 6E). Integration of LFD with LAMP-based microfluidic platform is a competitive

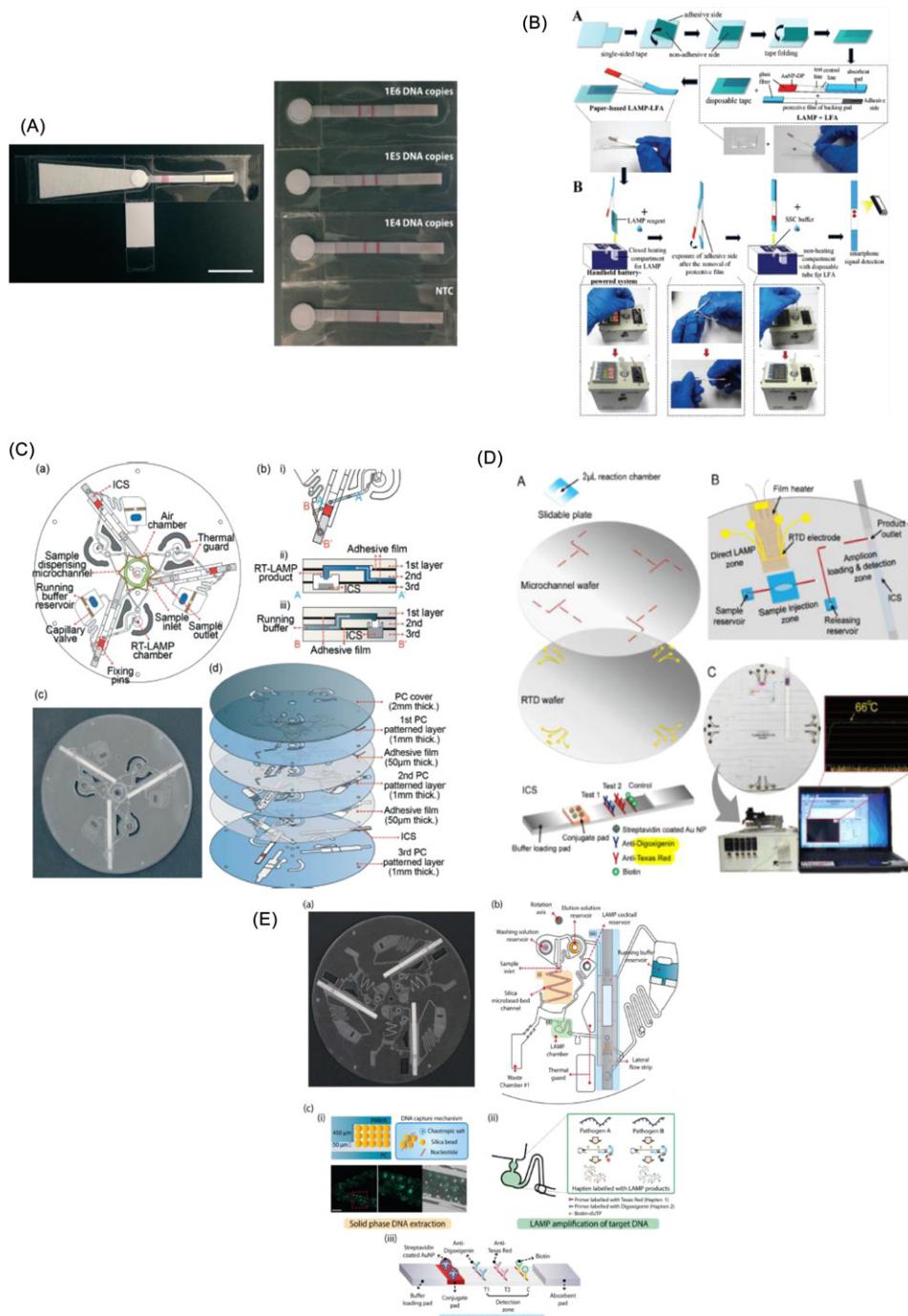
candidate for POCT since endpoint detection does not require any specific instrumentation and can provide high-throughput analysis. Nevertheless, the preparation of strips and the operation of installation are not only time-consuming, but also costly. Furthermore, this method could not provide quantitative analysis of the amplification products.

ELISA is a further immunoassay method for the detection of LAMP amplified products. Antigen-labeled nucleotides are directly incorporated into amplicons during the amplification process, amplicons later denature and hybridize to a specific captured DNA probe, and finally detecting labeled LAMP amplicons via ELISA. The LAMP-ELISA assays for detecting *Salmonella serogroup D* and *Salmonella* were obtained with LODs of 4 CFU/mL and 10 CFU/mL, which was 10 and 100 times more sensitive compared to PCR-ELISA and turbidity-based conventional LAMP method, respectively (Ravan and Yazdanparast 2012; Ravan and Yazdanparast 2013). This technique does not require expensive reader equipment and achieves better sensitivity. However, its drawbacks are: (1) the long experimental process, (2) requirement of professional training and (3) high risk of carry-over contamination. Lo et al. (2012) designed a paper-based device that has the capacity of detecting the dengue virus via RT-LAMP with a concentration of about 300 ng/mL. So far, studies about LAMP-ELISA based microfluidic chips have been rarely reported and the technique needs to be developed further.

## Real-time detection methods

### Real-time turbidity detection

Optical detection has been introduced to monitor the LAMP reaction process in real time. This can be divided into real-time turbidity methods and real-time fluorescence methods, according to whether dye is added or not. The real-time turbidity method utilizes the production of  $Mg_2P_2O_7$  precipitate as the byproduct of the LAMP reaction.  $Mg_2P_2O_7$  increases the turbidity of the sample and the result can be monitored

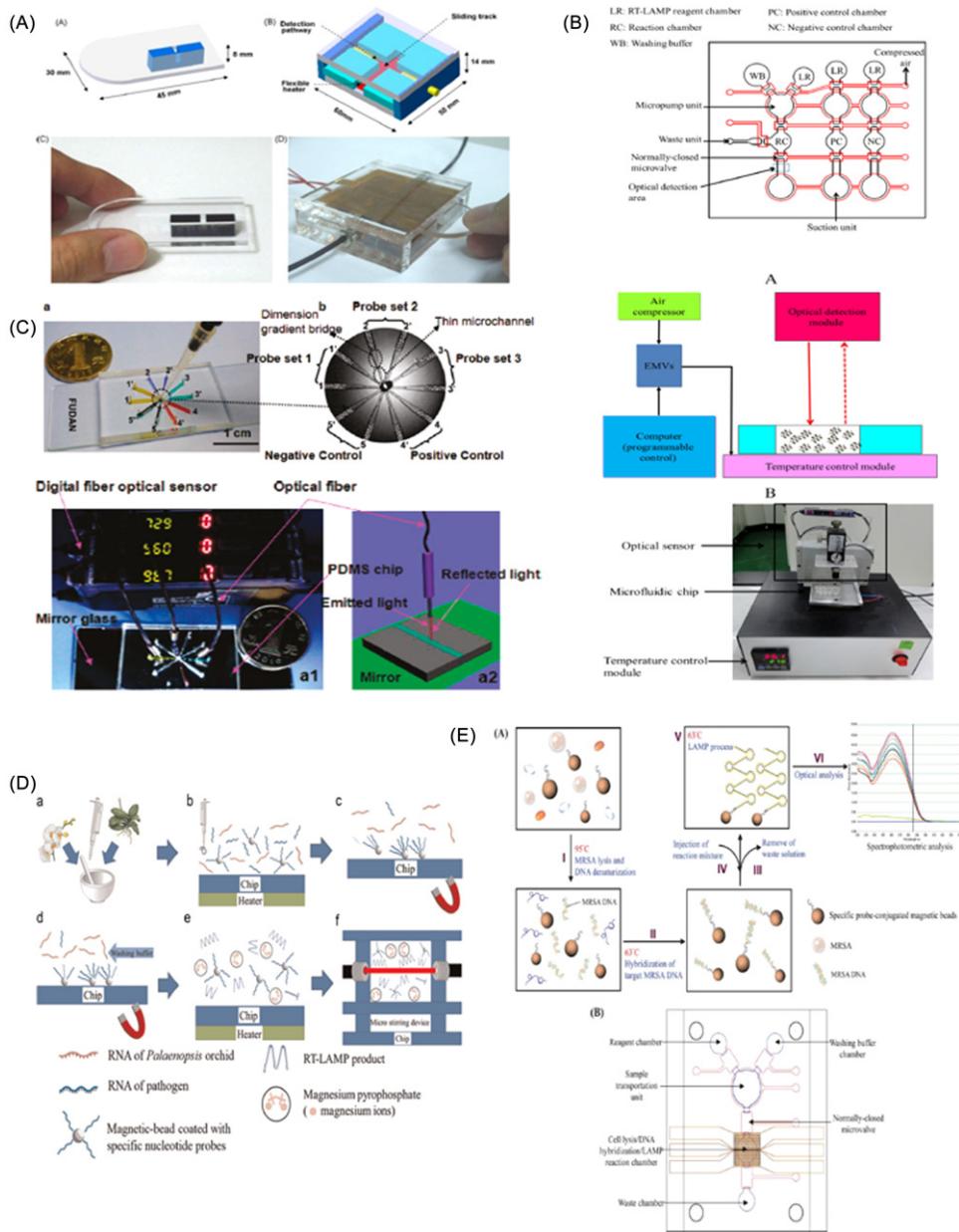


**Figure 6.** Schematic of LAMP-based microfluidic chips using immunoassay. (A) A fully integrated paperfluidic molecular diagnostic chip for the extraction, amplification, and detection of nucleic acids. (B) An integrated paper-based device incorporating LAMP and LFD. (C) Integration of RT-LAMP with LFD on a centrifugal microchip for influenza A virus detection. (D) The Direct LAMP-ICS microdevice which consists of a slidable plate, a microchannel wafer, an resistance temperature detector (RTD) wafer and an immunochromatographic strip (ICS). (E) Integration of LAMP with LFD on a centrifugal microchip for the detection of two targets simultaneously. Reprinted with permission from Refs. Rodriguez et al. (2016), Choi et al. (2016), Jung et al. (2015), Lee et al. (2016) and Park et al. (2017), respectively.

in real-time via optical instruments, including turbidimeters, optical fibers, and spectrophotometers. Gene copy number can be quantified by plotting turbidity against time to positive signal (threshold time). Lee et al. (2008) integrated a micro LAMP-reactor with a temperature-regulated optical detection unit to monitor the turbidity changes, enabling the detection of HBV DNA at a threshold level of 10,000 copies/mL within 60 min. The relationship between the turbidity values and the initial concentration of DNA template were highly correlated ( $R^2 = 0.9605$ ) (Fig. 7A). In another study,

Chang, Yang, Lin, et al. (2013) used a real-time turbidimeter to detect turbidity changes in the reaction chambers of a microfluidic chip, which consists of RNA extraction, purification, and transcription loop-mediated isothermal amplification (RT-LAMP) reaction units. The LOD is 5 pg for the detection of *Phalaenopsis* orchid viruses directly from fresh leaves (Fig. 7B).

An octopus-like LAMP-based microfluidic chip was developed to detect viruses in clinical samples using optical fibers. During the testing process, the emitted light from



**Figure 7.** Schematic of LAMP-based microfluidic chips with real-time turbidity detection apparatus. (A) Microchip integrated with turbidimeters for HBV detection. (B) Microchip integrated with turbidimeters for the detection of viruses from *Phalaenopsis* orchid leaves. (C) Microchip integrated with optical fibers to differentiate three influenza A subtypes. (D) Microchip integrated with buried optical fibers for *Phalaenopsis* orchid pathogens detection. (E) Microchip integrated with spectrophotometers for methicillin-resistant *Staphylococcus aureus* detection. Reprinted with permission from Refs. Lee et al. (2008), Chang, Yang, Lin, et al. (2013), Fang et al. (2011), Lin et al. (2015), and Wang, Lien, Wu, et al. (2011), respectively.

the fiber traverses the LAMP reaction chamber first; then, it is reflected by a mirror underneath the chip, and finally recorded by the digital sensor. The chip has 10 microchambers loaded with specific LAMP primers, each of which is connected to the corresponding thin microchannel via a dimension gradient bridge. It has the ability of simultaneously analyzing multiple genes with a LOD below 10 copies/ $\mu$ L within 30 min (Fig. 7C) (Fang et al. 2011). Lin et al. (2015) also designed a microfluidic platform capable of RNA purification, RT-LAMP reaction, and direct on-chip optical detection of the product. In this system, a horizontal buried optical fiber was used to detect  $Mg_2P_2O_7$ -induced turbidity changes, and a new micro-stirring device was integrated to evenly distribute the  $Mg_2P_2O_7$  precipitate. A

LOD of 25 fg was obtained for the detection of viral pathogens in *Phalaenopsis* spp. orchids within 65 min (Fig. 7D).

In addition, the detection for pathogen methicillin-resistant *Staphylococcus aureus* (MRSA) was achieved via an integrated magnetic bead-based microfluidic system with the incorporation of a spectrophotometer. This chip detected the target within 60 min with an LOD of 10 fg/mL, which is 1000 times more sensitive compared to conventional PCR (Fig. 7E) (Wang, Lien, Wu, et al. 2011). The real-time turbidity method has been considered as the easiest way of monitoring LAMP reaction, which has the capability of high automation and does not require specific probes. However, the main drawbacks are the inhomogeneity of particle size,

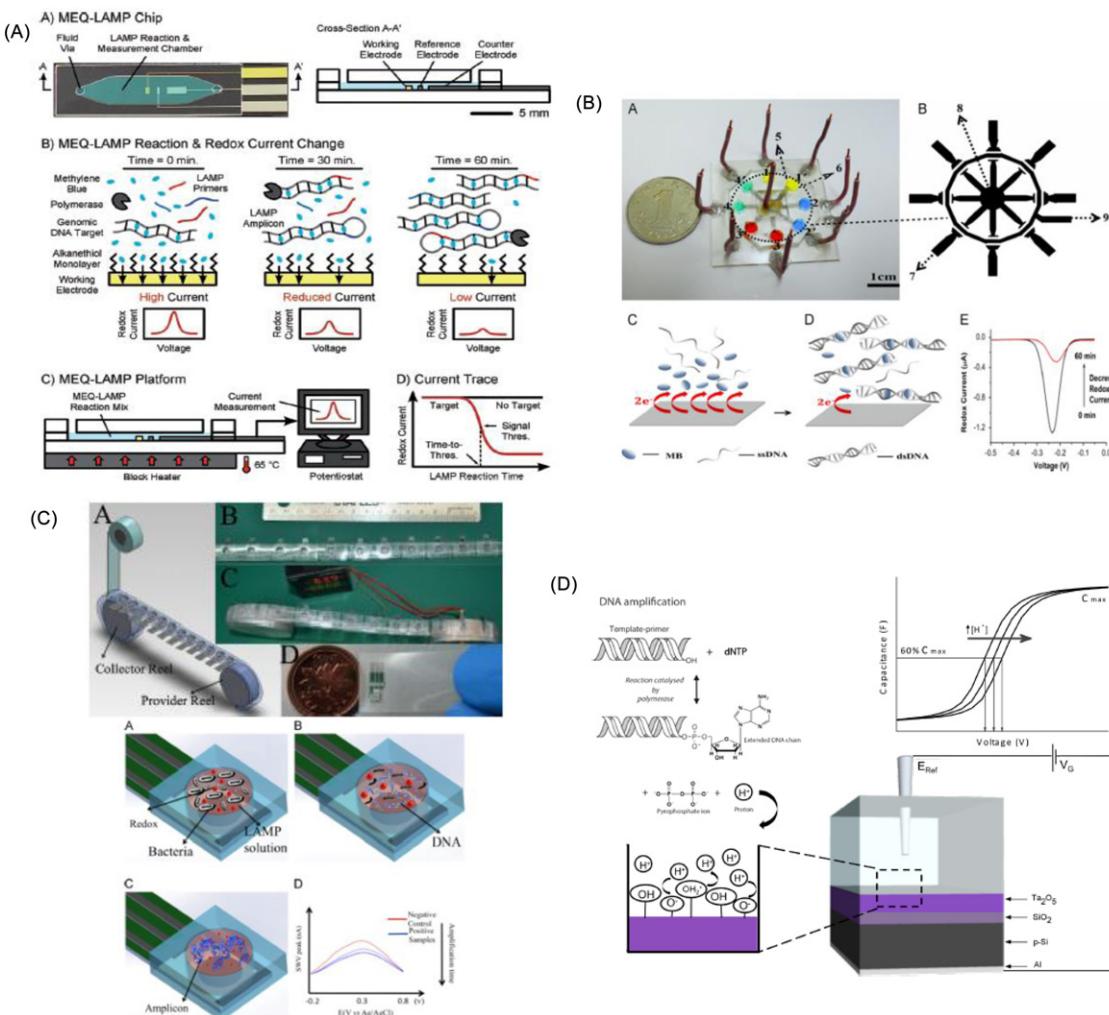


**Figure 8.** Schematic of LAMP-based microfluidic chips with real-time fluorescence detection apparatus. (A) Microchip integrated with CCD camera for waterborne pathogens detection. (B) Microchip integrated with custom-made, portable nucleic acid quantification device. (C) Microchip integrated with a disposable CMOS sensor module. (D) Microchip integrated with PMT detection. (E) Microchip integrated with ADP detection. (F) An endpoint detection using a blue LED and a cell phone camera. (G) A device for point of care genetic testing using a smartphone. (K) Digital microfluidic system for LAMP reaction using molecular beacon probes. Reprinted with permission from Refs. Ahmad et al. (2011), Liu et al. (2015), Wang, Kim, and An (2017), Chang, Yang, Wang, et al. (2013), Liu, Geva, et al. (2011), Liu et al. (2012), Stedtfeld et al. (2012), and Wan et al. (2017), respectively.

uneven spatial distribution, re-dissolution of  $Mg_2P_2O_7$  precipitate, and that the samples possess high turbidity.

### Real-time fluorescence detection

Real-time fluorescence is the most popular method due to its extreme sensitivity. The dyes used in the endpoint determination of the LAMP reaction can also be applied to real-time monitoring. With the help of a standard curve generated from different concentrations of gene copy number plotted against threshold time, the initial concentration of a sample can be extrapolated. The optical instruments, including charge coupled device (CCD), complementary metal oxide semiconductor (CMOS), photomultiplier tube (PMT), and avalanche photo diode (ADP), can be used to monitor the LAMP reaction in real-time. Typical examples are listed in this section. A CCD-based microfluidic chip was developed for the detection of waterborne pathogens using SYTO-82 dye. Compared to commercial real-time PCR instruments, the signal-to-noise ratio (SNR) was increased 8-fold and the threshold time ( $T_t$ ) was reduced from 2.7 to 9.8 min when the CCD exposure time increased to 5 s for  $10^5$  starting DNA copies. A single DNA copy for the *Campylobacter jejuni* 0414 gene was achieved with a  $T_t$  of 19 min (Fig. 8A) (Ahmad et al. 2011). Liu et al. (2015) integrated a RT-LAMP-based microfluidic chip with a handheld USB-based fluorescent microscope for monitoring the HIV virus in real-time. The excitation for the fluorescence provided with a blue LED, and the fluorescence imaging was collected via CCD camera, which transmits to a computer through a USB interface. The sensitivity of this method was smaller than 50 copies per sample (15  $\mu$ L) (Fig. 8B). In comparison to CCD, CMOS has shown great potential for integration and detection. Wang, Kim, and An (2017) designed a novel CMOS image sensor system for quantitative LAMP assays to detect foodborne pathogens. The LOD for the detection of pathogen *E. coli* O157:H7 was 10 fg/ $\mu$ L by using HNB dye and the assay time was within 40 min (Fig. 8C). Additionally, PMT and ADP are also popular photodetectors. A PMT-based microfluidic chip was developed for the detection of four different aquaculture pathogens, namely *Streptococcus agalactiae*, koi herpes virus, Irido virus, and *Aeromonas hydrophila*. The entire process could be performed within 65 min, including sample pretreatment, liquid transportation, washing process, LAMP amplification and real-time fluorescent detection via PMT. The LOD was 20 copies per sample (25  $\mu$ L) with the employment of SYTO-82 dye (Fig. 8D) (Chang, Yang, Wang, et al. 2013). Liu, Geva, et al. (2011) designed a single-chamber LAMP chip that utilizes a Flinders Technology Associates (FTA) membrane for DNA and/or RNA isolation, purification, and concentration without the need for elution. The reaction process was monitored in real time via portable ESE optical detector that consists of LED as the excitation light source and ADP for fluorescence detection. The LOD was 10 copies of HIV per saliva sample (20  $\mu$ L) with the employment of SYTO-9 dye (Fig. 8E). Furthermore, the LAMP chamber was further equipped with thermo-responsive PDMS valves to seal the



**Figure 9.** Schematic of LAMP-based microfluidic chips using real-time electrochemical detection. (A) A microfluidic electrochemical quantitative loop-mediated isothermal amplification (MEQ-LAMP) system for pathogenic DNA detection. (B) A microchip for differentiating multiplexing targets with eight isolated electrochemical microchambers. (C) A roll-to-roll cassette device integrated with SPE. (D) A pH-sensing electrochemical platform. Reprinted with permission from Refs. Hsieh et al. (2012), Luo et al. (2014), Safavieh, Ahmed, Ng, et al. (2014), and Veigas et al. (2014), respectively.

amplification reactor during the heating process (Liu, Mauk, and Bau 2011).

A LAMP-based microfluidic chip combined with a cell phone or iPod Touch will be more suitable for POCT. A sample-to-use microfluidic chip was designed for the rapid molecular identification of mosquito type. The device was integrated with a blue LED and a CCD camera from a cell phone, which excited the intercalating SYTO-9 dye and monitored the fluorescence signal respectively (Fig. 8F) (Liu et al. 2012). In a similar method, Stedtfeld et al. (2012) designed a valve-less microfluidic chip containing four arrays of 15 reaction wells to detect multiple genes, and raw data was obtained via iPod Touch and emailed to a personal computer for further analysis. Each reaction well was embedded with an LED light and optical fiber and the emitted fluorescence from each well was transferred to the photodiode through an optical fiber. An LOD of 13 copies per sample ( $1\text{ }\mu\text{L}$ ) was achieved using SYTO-81 dye (Fig. 8G).

But, the use of intercalating fluorescent dyes is prone to detection of nonspecific amplification, which increases the likelihood of false-positives results. Efforts have been made to discriminate nonspecific amplification using fluorophores-

labeled nucleic acids that hybridized to a specific single-stranded DNA structure, such as labeled primers (Kouguchi et al. 2010; Gadkar et al. 2018), alternatively binding quenching probes (Mashooq et al. 2016), fluorescence energy transfer-based probes (Ball et al. 2016; Jiang et al. 2015), hybeacons probes (Howard et al. 2015), and molecular beacons (Bakthavathsalam et al. 2018). Wan et al. (2017) developed a digital microfluidic platform that integrates sample preparation and fluorescence detection. To enhance the specificity of the LAMP reaction, low- $T_m$  molecular beacon probes were used in this system (Fig. 8K). Generally, real-time fluorescence method for monitoring the LAMP reaction is considerably faster and achieves higher sensitivity than that performed by a real-time turbidity; furthermore, it is less affected by the presence of opaque substances in the mixture. However, the inhibitory potential of the dyes and complicated probe designs should not be neglected.

### Real-time electrochemical detection

Real-time monitoring of the LAMP reaction is also achievable through *in-situ* electrochemical interrogation. Hsieh

et al. (2012) established an electrochemical LAMP microfluidic platform to detect *S. typhimurium* with a LOD of 16 copies/ $\mu\text{L}$  in less than 1 h. The microfluidic chamber contained a platinum counter, reference electrodes, and a gold working electrode. A MB indicator was used as electrochemical redox molecule. The real-time monitoring of DNA amplicons was achieved via integrated electrodes to measure decreases in current resulting from increasing binding of MB to LAMP reaction products (Fig. 9A). Similarly, Luo et al. (2014) designed an octopus-like microfluidic chip to detect three upper respiratory tract infection (URTI) related bacteria, namely *Mycobacterium tuberculosis* (MTB), *Haemophilus influenza* (HIN), and *Klebsiella pneumonia* (KPN), with LODs of 28, 17, and 16 copies/ $\mu\text{L}$  respectively. The chip contained eight micro-chambers, each of which connected to a counter electrode, a reference electrode, and an indium tin oxide (ITO) working electrode. The redox molecule MB was also used in this system (Fig. 9B). Additionally, Safavieh, Ahmed, Ng, et al. (2014) designed a novel roll-to-roll ribbon device to detect bacteria in real-time using electrochemical square wave voltammetry (SWV). The device consisted of 12 reservoirs with flexible carbon screen-printed electrodes (SPE) attached to their bottom. The electroactive reagent in this system was Osmium redox ( $\text{Os}[(\text{bpy})_2\text{DPPZ}](\text{PF}_6)_2$ ), which does not inhibit LAMP and shows high binding activity. The detection of 30 CFU/mL of *E. coli* (in the range of  $30\text{-}3 \times 10^7$  CFU/mL) and 200 CFU/mL of *S. aureus* (in the range of  $200\text{-}2 \times 10^5$  CFU/mL) were achieved in both real-time and end-point detection (Fig. 9C).

During the electrochemical monitoring process, an ideal electroactive reagent should be chemically stable, preferentially bind to target dsDNA amplicon, and not inhibit the amplification. Nevertheless, almost all types of redox active compounds that exist defect partly. For example, Hoechst 33258 significantly inhibits the LAMP reaction and is therefore not suitable for real-time monitoring. MB exhibits low binding activity to dsDNA in comparison to other redox molecules. In contrast, ruthenium hexamine (RuHex) is more suitable for real-time electrochemical detection since it lacks intercalating ligands and binds electrostatically with the anionic dsDNA backbone. Ahmed et al. (2013) employed this as an indicator to monitor LAMP amplicons without any immobilization of probes onto the electrode surface. The LODs were 30 copies/mL and 20 copies/mL, respectively, for *S. aureus* and *E. coli* in less than 30 min. Additionally, Zhang et al. (2011) developed a voltammetric method to monitor the LAMP reaction based on the electrochemical response of 2'-deoxyguanosine 5'-triphosphate (dGTP) at a carbon nanotube array electrode (CNTE). With this strategy, inhibition is not a concern as no auxiliary indicators are used.

The pH-sensing electrochemical platform is a further label-free method to monitor the LAMP reaction in real-time. The pH of the solution changes, either due to hybridization or amplification reaction, which yields a local pH variation and a rearrangement of ionic species near the sensor surface, which can then be monitored via electrochemical or electrical sensing. This approach avoids the need for

redox molecules in the LAMA reaction and/or time-consuming electrode functionalization with a recognition probe. Xie et al. (2014) used a pH meter to measure the released hydrogen ions ( $\text{H}^+$ ) during the LAMP process. Veigas et al. (2014) also developed field-effect-based sensor made of tantalum oxide ( $\text{Ta}_2\text{O}_5$ ) combined with an AgCl reference electrode to monitor  $\text{H}^+$  in the reaction solution. Although the sensitivity of the developed device was slightly lower than that using real-time SYBR Green fluorescence method, it has almost no running cost. The target cMYC proto-oncogene was quantified in the range of  $10^3$  to  $10^{11}$  copies/mL (Fig. 9D).

In general, electrochemical sensing combined with LAMP-based microfluidic chip offers compelling advantages due to its easy manipulation, high sensitivity, and low cost. However, many factors affect the electrochemical signal, such as electrode material, pH, and ionic strength of the solution. The installation of electrodes into the reaction mixture may increase the potential for cross-contamination. Furthermore, the "signal-off" mode, which the target binding leads to the decrease of signal output, suffers from the limited signal capacity and could cause false-positive results. But with the overcoming of these challenges, we are optimistic that further real-time electrochemical LAMP chip will possess more accurate capability to detect pathogens.

### Other real-time detection methods

Surface plasmon resonance (SPR) sensor have been used for real-time LAMP detection, as the number and size changes of solute molecules alter the refractive index of bulk solution. Chiu et al. (2012) constructed an SPR-LAMP chip where a single layer graphene film was immobilized on the Au surface for detecting *Tuberculosis bacillus* (TB) DNA. In another study, Chuang et al. (2012) developed a SPR-LAMP system integrated with a PMMA micro-reactor and a polycarbonate (PC) prism coated with a 50 nm Au film. This microchip can detect hepatitis B virus (HBV) template mixed in the 10  $\mu\text{L}$  LAMP solution in 17 min and the LOD was 2 fg/mL. The label-free SPR method has the advantages of rapid monitoring, real-time analysis, and low sample volume required; however, it usually requires bulky and expensive readers and the precision could be further improved.

The bioluminescence real-time assay is based on the dynamic changes of released pyrophosphate during the DNA synthesis that emits light via serial catalytic reactions. Quantification of the starting target DNA is achieved by measuring the time it needs to reach peak bioluminescence, which greatly simplifies the data interpretation and hardware requirements. Kiddle et al. (2012) reported the applicability of this method with LAMP reaction for determination of genetically modified (GM) maize DNA at low levels of contamination (0.1–5.0% GM).

### A comparison between different methods

In this article, we provided an overview of the recent advances in LAMP-based microchip technology for detecting

**Table 5.** Comparative features of monitoring methods for LAMP.

Methods		Advantages	Disadvantages	Need device/Cost	Applied frequency
End-point detection methods	Turbidity	Simplicity, rapidly	Subjective error, high detection limit	No/No	Medium
	Dyes	Simplicity, relatively high sensitivity	Inhibition of amplification	Now/Low	High
	Electrochemistry	High sensitivity, fast response	Inhibition of amplification, cross-contamination	Yes/Low	High
	Electrophoresis	Reporting length of amplicons, high sensitivity	Labor-intensive post-amplification process	Yes/Medium	Low
	Immunoassay	High specificity, special equipment not required	Labor-intensive process, cross-contamination	No/High	Medium
Real-time detection methods	Turbidity	Automation, probe/indicator-free	Homogeneity and high transparency demand	Yes/Low	Medium
	Fluorescence	Automation, fast response, free from contamination, high sensitivity	Inhibition of amplification, interference from background fluorescence	Yes/Medium	High
	Electrochemistry	Automation, unaffected by sample opacity, high sensitivity	Inhibition of amplification, cross-contamination, low reproducibility	Yes/Low	High
	SPR	Probe/indicator-free, high sensitivity	Expensive reader	Yes/High	Low
	Bioluminescence	Simplicity, rapidly	Specific enzyme	No/Medium	Low

pathogens, based on end-point and real-time determination mechanisms. All the methods presented above can be used to achieve monitoring of the LAMP reaction in a variety of situations. **Table 5** summarized the distinct features between them. Overall, real-time detection methods are capable of quantitative monitoring, which is more practicability than end-point detection methods. Amongst them, those based on optical and electrochemical techniques have been in the forefront. Furthermore, electrochemical techniques are superior to optical techniques, due to their higher sensitivity and also non-necessity of optical-electrical signal transferring components in the device. In the near future, it is envisioned that an ideal monitoring method may provide an optimal LAMP-based microchip for point-of-care diagnosis if these drawbacks can be properly addressed.

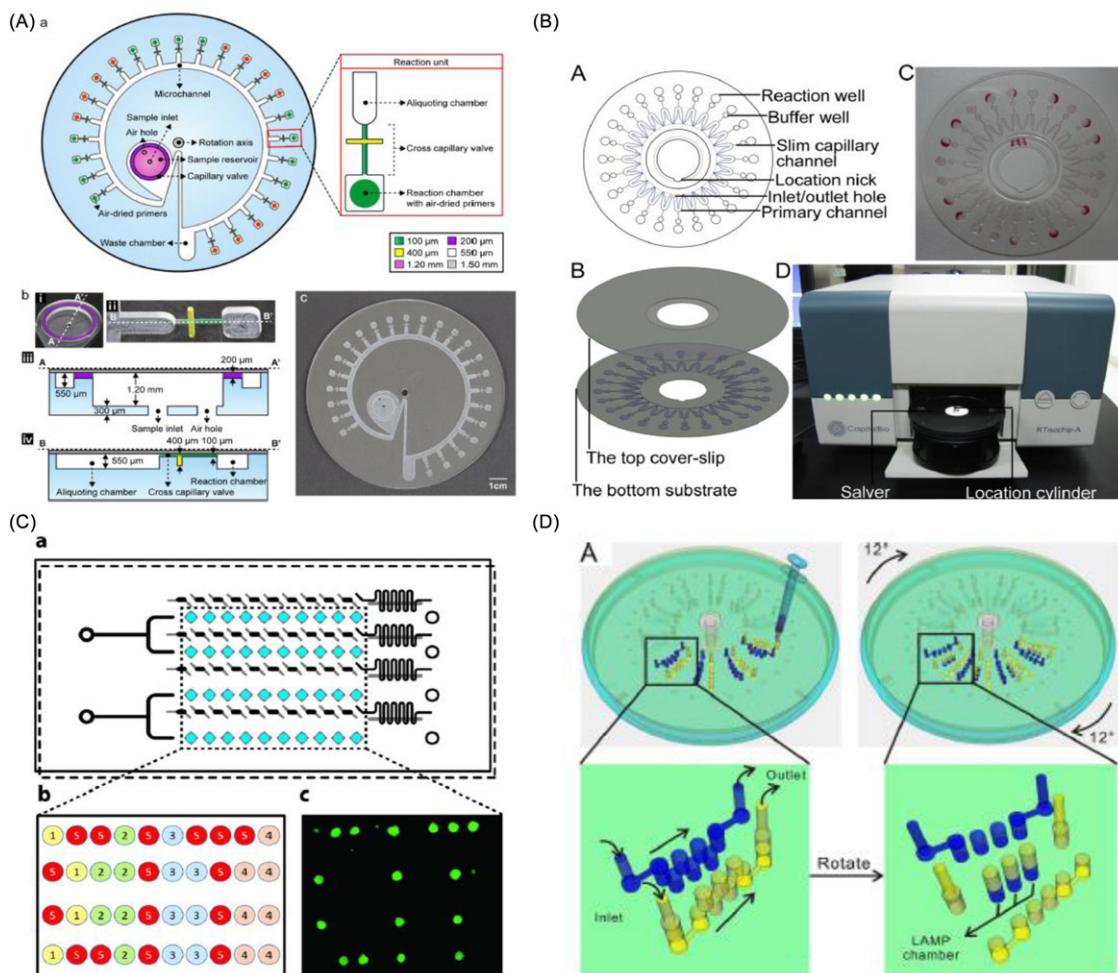
## Future promoting aspects

### Multiplex analysis

The capabilities of LAMP-based microfluidic chips for multiplexing and high-throughput screening are satisfactory for many research fields. Various forms of detection of the LAMP reaction have been reported for simultaneously detecting multiple pathogens, such as centrifugal microfluidic platform, compact disc, and SlipChip.

The centrifugal microfluidic platform is an outstanding system where liquid handling and control can be automatically operated via rotational speed, and it is insensitive to liquid properties, such as viscosity, pH, or wetting behavior. Seo et al. (2017) developed a centrifugal microsystem where the LAMP reaction was performed in 24 reaction chambers to identify three types of foodborne pathogens, i.e., *E. coli* O157:H7, *Salmonella typhimurium*, and *Vibrio parahaemolyticus*. EBT dye was used to confirm the target amplicons by observing the color change with the naked eye (**Fig. 10A**). Similarly, Zhou et al. (2014) constructed a microfluidic chip to detect 10 pathogenic bacteria in aquatic animals. A LOD of 0.28–4.56 pg/µL was achieved in less than 30 min by

implementing real-time and endpoint fluorescent intensity measurement (**Fig. 10B**). However, real-time sensing is difficult on centrifugal platforms because the disc is spinning while the assays are performed. Furthermore, the compact disc can perform several hundreds of parallel assays on one chip. For example, Santiago-Felipe et al. (2016) used a micro-reactor embedded compact disc to conduct DNA amplification, and the real-time target determination was realized via optical scanning with a disc drive. Ninety-six samples could be simultaneously analyzed in 15 min with a LOD of 5 CFU/mL. The main drawbacks are the complexity of fabrication and that high-precision modalities are required for flow controlling and detection. SlipChip is also a novel microfluidic platform with the ability to perform multiplexed solution-phase experiments in a simple format. The SlipChip consists of two plates. The bottom plate chambers are filled with reagents and ducts and the top plate wells are loaded with sample. Afterwards, the two preloaded plates are brought in contact so that the wells in them contact each other, which induces the mixing of reagents and sample (Du et al. 2009). Sun et al. (2013) used a SlipChip microfluidic device to evaluate the suitability of digital RT-LAMP (dRT-LAMP) for quantification of HIV viral RNA. They reported that the efficiency of two-step dRT-LAMP, in which the reverse transcription of RNA molecules was fully independent with LAMP reaction, could be increased 10-fold compared to one-step dRT-LAMP. The same group further combined this method with a cell-phone imaging system to monitor the reaction in real-time (**Fig. 10C**) (Selck et al. 2013). In another study, conventional SlipChip was reformed to a hybrid rotational SlipChip system where one-step rotation allowed immediate mixing and reaction of samples with reagents. This SlipChip showed a LOD of 7.2 copies/µL for *Vibrio fluvialis* and realized simultaneous identification of five pathogenic bacteria on one single chip in 60 min (**Fig. 10D**) (Xia et al. 2016). Although SlipChip performs multiplexed reactions without pumps or valves, the fabrication is complex and gaps exist between both plates.

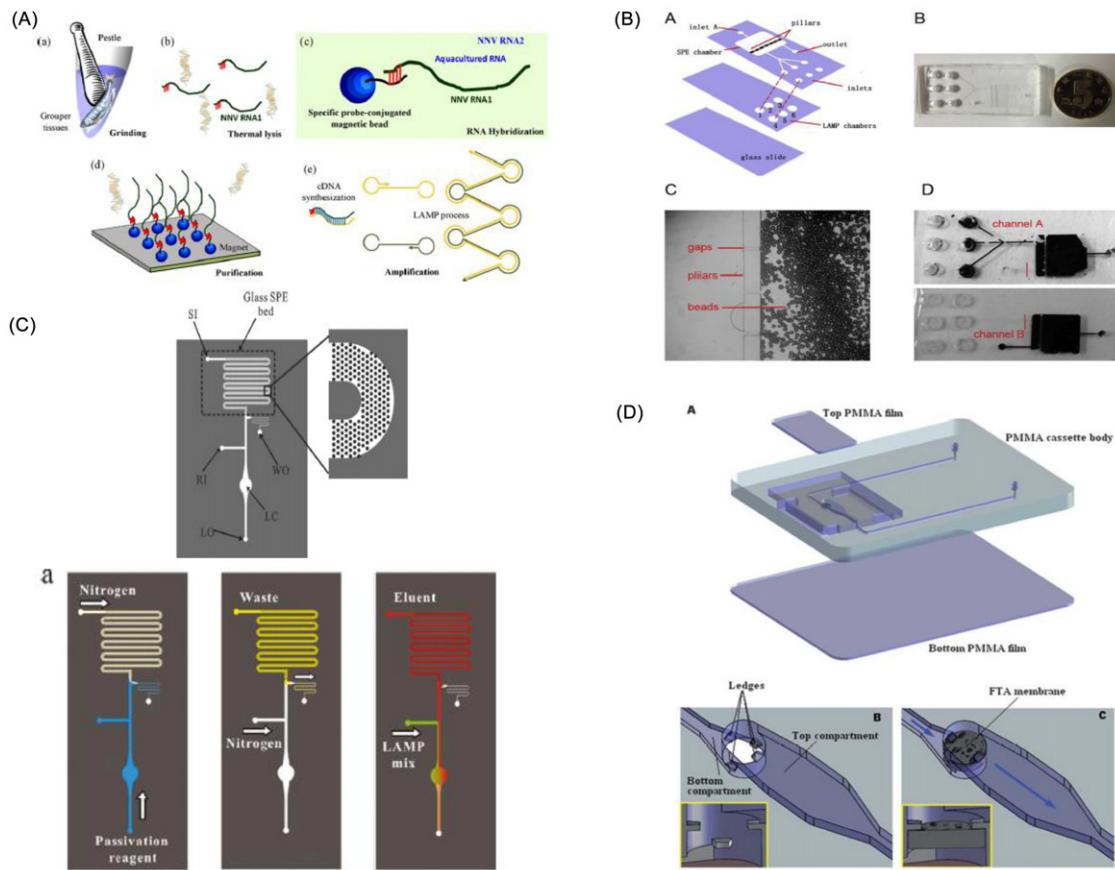


**Figure 10.** Multiplexing and high-throughput LAMP-based microfluidic chips. (A) A centrifugal LAMP microchip with 24 reaction units for detecting three kinds of foodborne pathogens. (B) A centrifugal LAMP microchip with 24 reaction units for detecting ten pathogenic bacteria in aquatic animals. (C) A SlipChip using five different primer sets to detect HIV-1 virus. (D) A rotate & react SlipChip (RnR-SlipChip) for detecting five pathogenic bacteria. Reprinted with permission from Refs. Seo et al. (2017), Zhou et al. (2014), Selck et al. (2013), and Xia et al. (2016), respectively.

As mentioned above, the strategy of detecting multiple targets is typically pursued to apply parallel channels or chambers on one chip. However, for detecting multiple targets in one single channel or chamber, the challenging issue can be addressed through tagging a series of target-specific barcodes or diverse fluorophores. For example, four types of pathogens (HBV, HCV, HIV, and TP) could be simultaneously detected via multiplex LAMP reaction following nicking reaction and pyrosequencing, in which target amplicon was tagged with a specific barcode (Liang et al. 2012). Additionally, a multiple endonuclease restriction real-time LAMP (MERT-LAMP) assay was developed to detect multiple DNA targets. In this system, the LAMP primer (FIP and/or BIP) contains 5' end short sequences with an endonuclease recognition site, and the new LAMP primer was modified at the 5' end with a fluorophore as well as a dark quencher (Wang et al. 2015). If detection of multiple targets can be accomplished in a single chamber, the chip dimension, the sample/reagent consumption and energy requirement could be greatly reduced. Therefore, further research is needed.

### Integration assay

The fully integrated LAMP-based microfluidic chips, that perform all the necessary steps from sample preparation to target detection, are particularly suitable for resource-limited settings and many fields where on-site detection is necessary. Here, the sample pretreatment process should be integrated on a single microchip. Many materials suitable for the extraction of nucleic acids have been reported, including modified magnetic bead, silica bead/sol gel solid-phase extraction (SPE) bed, silica/glass pillars, and the Flinders Technology Associates (FTA) membrane. For example, an integrated microfluidic system consisting RNA extraction and RT-LAMP process was developed to detect nervous necrosis virus (NNV) from fish tissue. During the extraction process, the specific probe-conjugated magnetic beads were utilized to recognize and hybridize target RNA. The sensitivity of this system is about 100-fold higher than conventional RT-PCR and the entire analysis protocol can be completed within 60 min (Fig. 11A) (Wang, Lien, Wang, et al. 2011). In a similar approach, the rapid identification of MRSA was achieved with a LOD of 10 fg/mL (Wang, Lien, Wu et al.

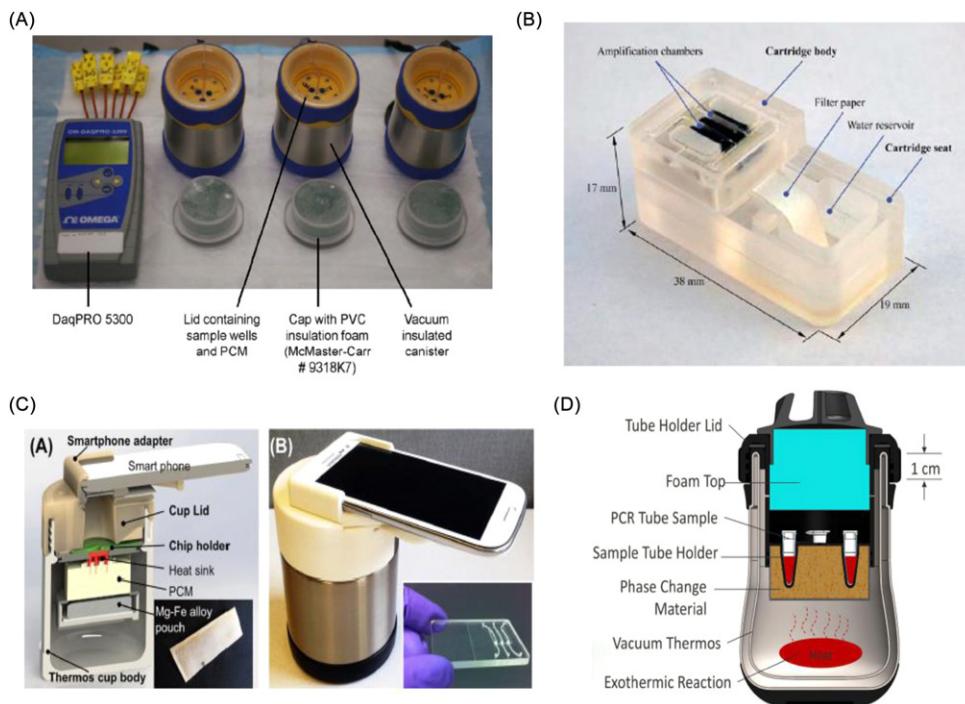


**Figure 11.** Schematic of LAMP-based microfluidic chips integrated with pretreatment process. (A) The purification of target RNA samples using specific probe conjugated magnetic beads. (B) The extraction of DNA using silica-based SPE method. (C) The extraction of DNA using serpentine channel where micropillars were etched. (D) A cassette installed with FTA membrane. Reprinted with permission from Refs. Wang, , Lien, Wang, et al. (2011), Guo et al. (2015), Wu et al. (2011), and Liu, Geva, et al. (2011), respectively.

2011). The silica-based solid phase extraction (SPE) method is an efficient sample preparation process, which relies on the interaction between DNA and the silica beads in the presence of a high chaotropic agent. The extracted DNAs are eluted with a low-salt buffer and concentrated into a small volume. Guo et al. (2015) designed a LAMP-based microfluidic chip that integrated this method for bacterial detection. The silica-based SPE chamber was installed prior to LAMP chambers to isolate bacterial DNA by binding to silica surface. Three bacterial strains (*E. coli* O157:H7, methicillin-resistant *Staphylococcus aureus*, and methicillin-sensitive *Staphylococcus aureus*) were detected in less than 2 h with a LOD of 100 CFU/100 mL (Fig. 11B). Silica or glass pillars can also be integrated into microfluidic channels to extract nucleic acids. A glass microchip, consisting of nucleic acids extraction region and LAMP chamber, was developed to detect target DNA utilizing SYBR Green I dye. A serpentine channel with an array of glass micropillars was designed to increase the channel surface area and the capture efficiency of nucleic acids. The whole analysis could be finished within 2 h (Fig. 11C) (Wu et al. 2011). Additionally, the FTA membrane is a chemically-treated filter paper enabling the rapid cell lysis, nucleic acids isolation, and partial purification. The nucleic acids captured by the membrane are directly used as templates for amplification without elution, thus simplifying the assay flow (Labarre et al. 2011).

Liu, Geva, et al. (2011) developed a single-chamber cassette to detect the HIV virus that integrates a FTA membrane to capture HIV RNA. The assay showed a LOD of 10 copies/20  $\mu$ L within 1 h (Fig. 11D).

Until now, LAMP-based nucleic acid amplification detections still require electricity to achieve amplification temperatures (grid, battery, thermocycler, heat block, or water bath). To eliminate the need of electrical power, several researchers have proposed exothermic chemical reaction-based heating for isothermal amplification. For example, Curtis et al. (2012) reported a non-instrumented heating device for HIV-1 detection that generates heat from the exothermic reaction of calcium oxide and water (Fig. 12A). In another study, a self-heating cartridge was developed to detect *Escherichia coli*, replacing calcium oxide with magnesium iron alloy (Mg-Fe alloy) for the exothermic reaction. The heat is generated via magnesium reacting with water in the presence of iron, and the amplification temperature is regulated with the aid of a phase change material (Fig. 12B) (Liu, Mauk, et al. 2011). Based on this heating mechanism, the same group successfully designed a smart cup for the rapid and quantitative detection of pathogens and the fluorescence signal was recorded via smart-phone (Fig. 12C) (Liao et al. 2016). Similarly, Singleton et al. (2014) proposed an electricity-free platform to supply heat for the amplification reaction of HIV-1 RNA and a LOD of 6 copies/ $\mu$ L was



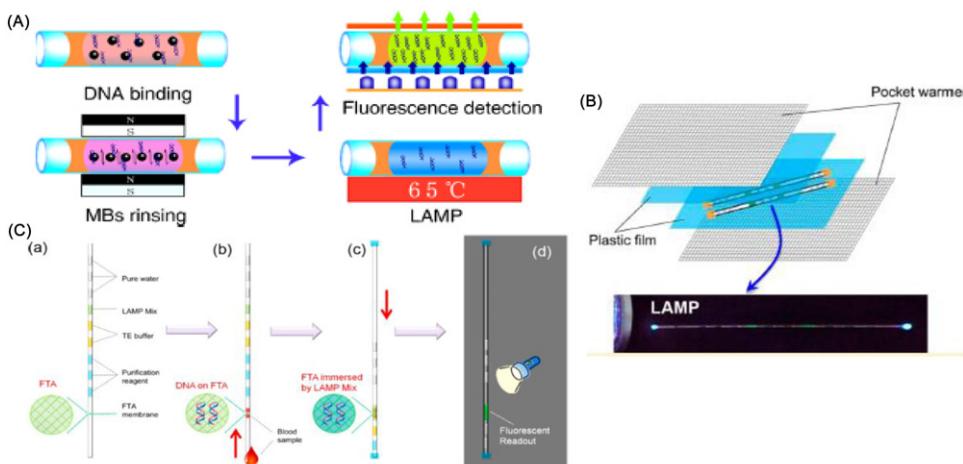
**Figure 12.** Electricity-free heating devices for on-chip LAMP amplification. (A) A portable non-instrumented nucleic acid amplification (NINA) device that uses calcium oxide for heat generation. (B) A self-heating cartridge that uses magnesium iron alloy for heat generation. (C) A minimally-instrumented, smart cup platform for molecular diagnostics that uses magnesium iron alloy for heat generation. (D) A NINA cartridge for HIV-1 detection that uses magnesium iron alloy for heat generation. Reprinted with permission from Refs. Curtis et al. (2012), Liu, Mauk, et al. (2011), Liao et al. (2016), and Singleton et al. (2014), respectively.

achieved using the LFD method (Fig. 12D). Moreover, a pocket warmer as heat source also can be used for the LAMP reaction. Hatano et al. (2010) developed a LAMP anthrax detection system that uses a disposable pocket warmer without the need for electricity. H1N1 was detected using an RT-LAMP kit with a pocket-warmer (Hatano et al. 2011). With the further modifications and improvements of technology, the fully integrated LAMP-based microchip can conduct all necessary steps from the sample to find out whether it is particularly suitable for field application.

### Capillary tube platform

Until now, most of the presented microfluidic chips (made by PDMS) still rely on fabrication in specialized facilities, resulting in high production costs. Additionally, the porosity and gas permeability of PDMS materials may obstruct the LAMP amplification due to bubble formation or vapor evaporation in PDMS-based microchips. To overcome these limitations, the capillary-based LAMP detection method has been developed with minimal fabrication complexity and simple parallelization. Liu et al. (2013) developed a capillary-array microsystem for detecting *Mycobacterium tuberculosis*, including DNA extraction, amplification, and fluorescence detection zones. During the analysis, magnetic beads (MBs) were utilized for DNA extraction and purification in sample droplets. After DNA purification, the sample was transported into the reaction zone for DNA amplification and was then quantified via fluorescence detection using SYTO-81dyes, which were added to the reaction mixture prior to the amplification. The integrated microsystem could process 10 samples in parallel achieving a LOD of 10 bacteria within

50 min (Fig. 13A). In another study, Zhang, Zhang, Sun, et al. (2014) used a capillary-based detection platform to simultaneously detect two influenza A virus (H1N1 and Flu A) with LODs of 2 copies of DNA. The capillary tube was loaded with reaction mixture via capillary action, then using segments of water droplets to isolate each reaction zone as well as to prevent contamination and applying a hand-held flashlight to read the fluorescent signal. The analytic process did not require external power supply and equipment (Fig. 13B). They further integrated the FTA membrane into microcapillary to capture and concentrate nucleic acids, thus simplifying sample pretreatment steps. The FTA card was picked up via syringe needle and pushed into the microcapillary using an ejector pin. Then, different segments of purification reagent, TE buffer, LAMP reaction mix, and water droplets were successfully loaded from the other end. During analysis, 0.2  $\mu$ L of untreated blood sample was injected from the end contained FTA card and was immersed in it for at least 1 h. After sequential washing of purification reagent and TE buffer, the FTA card was incubated in a LAMP reaction mix for amplification. Finally, the ends of the microcapillary were sealed via reagent/water droplets, air, and epoxy glue to avoid contamination, and the amplified target was detected via hand-held UV-flashlight. The assay time for detecting single-nucleotide polymorphisms (SNPs) in CYP2C19 from blood was 150 min (Fig. 13C) (Zhang, Zhang, Wang, et al. 2014). To a certain extent, a capillary-based LAMP detection platform could avoid the complex operation and its smooth inner surface could repress the formation of bubbles during the heating process. However, sealing methods are either highly complex



**Figure 13.** Schematic of LAMP-based capillary tube platform. (A) A capillary-array microsystem integrated with DNA extraction, LAMP reaction, and fluorescence assay for *M. tuberculosis* detection. (B) A microcapillary-based LAMP for multiplexed nucleic acids analysis. (C) Integrated capillary LAMP with preloaded reagents for nucleic acid pretreatment, amplification, and detection. Reprinted with permission from Refs. Liu et al. (2013), Zhang, Zhang, Sun, et al. (2014), and Zhang, Zhang, Wang, et al. (2014), respectively.

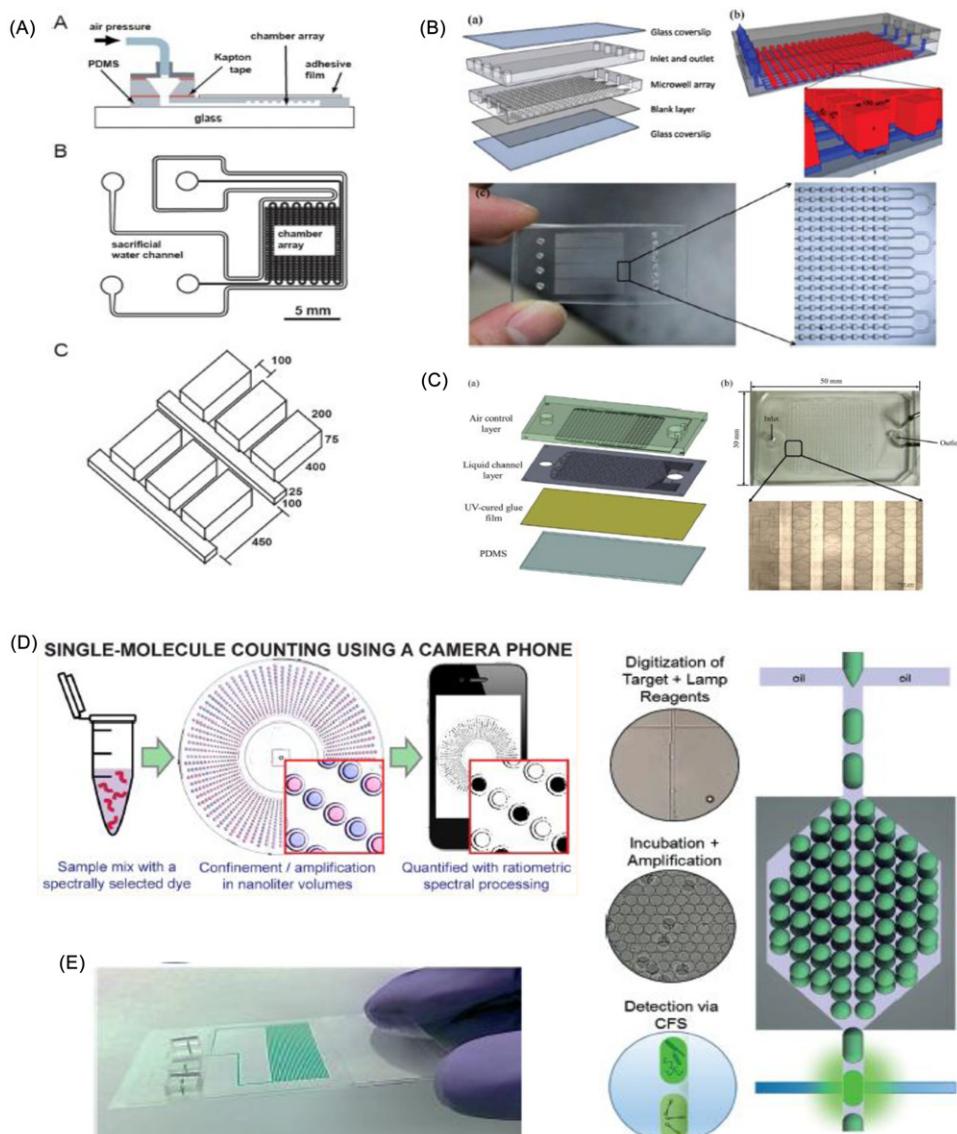
or ineffective, and an external pumping system or heating block is still required.

### Digital LAMP

Digital nucleic acid detection is becoming a popular technique for the accurate quantification of DNA or RNA copy numbers within a sample. This relies on separating the reaction volume into thousands of droplets, each of which contains less than one template molecule. After amplification, the number of droplets showing positive signal equals the number of templates in the original sample. Compared to the digital PCR (dPCR), the detection capability of digital LAMP (dLAMP) is more sensitive and faster. Its use can avoid the need for thermal cycling and precise temperature control. Various microfluidic platforms have been proposed for the practical implementation of the dLAMP technique, which can broadly be classified into two different types, i.e. static microwell arrays and continuous flow droplets. The first type involves fabrication of a large array of microwells on a substrate. For example, Gansen et al. (2012) designed a sample self-digitization (SD) platform which was the first microchip that integrated with dLAMP module. Based on the interplay between fluidic forces and interfacial tension, droplets were formed in the chip through manual/automated syringe pumps or external air pressure, which demonstrated sufficient size homogeneity with small variation (Fig. 14A). Alternatively, a self-priming compartmentalization (SPC) microchip for dLAMP was reported. The SPC chip consisted of four separate panels and each panel contained 1200 chambers with 6 nL volume. After a vacuum degassing step, the pressure difference of the air dissolved in PDMS provided the driving force for the liquid and oil to be sequentially get sucked into the chip. The system avoids the use of any external power source for transportation and control manipulation; however, the self-priming capability was only achievable when using a vacuum, which may be a restriction for long-term preservation (Fig. 14B) (Zhu et al. 2012). To eliminate this limitation, Ma et al.

(2018) developed a self-driven PDMS microchip that is surface-coated with a hydrophilic film. Due to the excellent hydrophilic properties, the sample mixtures could migrate through the microwells via capillary forces. Specific gene detection for vancomycin-resistant *Enterococcus* was achieved with a LOD of 11 copies/30 µL and an amplification time of 30 min (Fig. 14C). Moreover, a rotational SlipChip platform was also proposed for nucleic acid detection using a smart-phone camera as visual readout. Colorimetric analysis was used to exploit the camera detection while the LAMP reaction was performed in 5 nL wells (Fig. 14D) (Rodriguez-Manzano et al. 2016). In short, static microwell arrays offer great advantages in partition accuracy, reproducibility, and time-effectiveness compared to other digitized strategies. However, the requirement for chip design is high and the fabrication process is complex. In addition, the sample volume and well size are fixed by the chip structure, hindering its applicability.

Droplet-based microchips are also a promising method for dLAMP assay, where a large number of uniform droplets (nanoliter- to picoliter-sized) are generated in the oil phase via microemulsion. Each droplet contains LAMP reagent as well as target nucleic acid molecule. For instance, a continuous flow dLAMP-based microchip was developed for the detection of *Neisseria gonorrhoeae* with a LOD of 600 copies/µL, integrating droplets generation, incubation region, and fluorescence detection region in one chip. During testing, the sample mixtures were first digitized into picoliter-sized droplets, and then flowed into the serpentine chamber for incubation, which enabled the droplets to flow side by side. Finally, a narrow channel propelled droplet flow to the detection region, where fluorescence detection is conducted using confocal fluorescent spectroscopy microfluidic (Fig. 14E) (Rane et al. 2015). Collectively, droplet-based dLAMP provides high screening efficiency and the formation of droplets in oil phase could prevent potential cross-contamination and nonspecific amplification. The main drawback is that droplets are likely to break up during transfer and heating, leading to confusing results. In summary, integrating



**Figure 14.** Schematic of digital LAMP platform. (A) A sample self-digitization (SD) microchip for performing dLAMP. (B) A self-priming compartmentalization (SPC) microchip for performing dLAMP. (C) A self-driven microchip coated with a hydrophilic film for performing dLAMP. (D) A SlipChip-based dLAMP imaged by unmodified camera phones. (E) A droplet-based microchip for continuous flow dLAMP analysis. Reprinted with permission from Refs. Gansen et al. (2012), Zhu et al. (2012), Ma et al. (2018), Rodriguez-Manzano et al. (2016), and Rane et al. (2015), respectively.

dLAMP into sample-to-answer microfluidic chip offers great potential to achieve rapid and sensitive detection.

### Conclusion and prospects

LAMP is a very robust, innovative, and powerful gene amplification approach that eliminates the need for thermal cycling, where alternating extension and strand displacement reactions happen under isothermal conditions. Due to its excellent performance, LAMP is particularly suitable for miniaturization compared to traditional PCR. The integration of LAMP technique in microfluidic devices has shown great potential for the assay of molecular biology diagnosis, which reduces costs, total analysis time, and potential sources of contamination. In this review, we categorized the current LAMP-based microfluidic devices based on their detection mechanism and the most relevant examples were

presented. Whether the end-point or real-time monitoring for microchip systems are concerned, optical and electrochemical-based techniques are dominating the field. Moreover, further novel techniques such as immunoassay, SPR, giant magnetoresistive (GMR), and bioluminescence have also been developed as ideal detectors for LAMP reaction. However, there is still much room for improvement. Firstly, the LAMP-based microchip should integrate the full workflow from sample treatment to target detection (i.e., nucleic acid extraction, purification, pre-concentration amplification, and detection). Secondly, the potential cross-contamination and nonspecific amplification during the LAMP reaction easily produce false-positive results. dLAMP represents a highly sensitive and reliable quantification means to detect target nucleic acid, thus overcoming these deficiencies to a certain degree. Thirdly, selective detection of viable cells can be realized via pretreatment of samples with propidium

or ethidium monoazide (PMA/EMA) dye. This procedure has been used in conjunction with LAMP reaction; however, until now, it has not been successfully integrated into a microchip. Fourthly, the sophisticated design of integrated and automated devices is a challenge, and the need for micro-pumps and micro-valves complicate the chip fabrication process. Fifthly, the cost and energy consumption of devices are also important considerations, particularly for the applications in resource-limited settings or remote areas. Paper- and capillary-based microfluidic platforms are attractive substrates to carry out LAMP amplification for developing simple, low-cost molecular assays. Electricity-free cartridges based on exothermic chemical reactions reduce the dependence on electrical power supply and provide accurate testing without access to professional laboratories. Furthermore, these assays are expected to realize multiplex detection or supply personalized customization services if different primers are loaded in different reaction chambers on the chip. Finally, the ultimate automation design for a microchip is one in which several parallel reactions can be run, and dry reagents, primers, and enzymes are all pre-stored on the chip. Although this strategy could reduce the user operational error, its transport and storage require cold-chain management, as the LAMP enzymes and other chemical components cannot tolerate warm or even room temperatures. Furthermore, the results of LAMP amplification can be read by any unmodified smartphone camera (e.g. iphone or ipad), thus systematic multidisciplinary knowledge is urgently required for the realization of a truly portable and rapid detection device.

In general, the currently existing problems have become the most important issues for its practical application, and the road to the optimization is still long. Ultimately, it is worth pointing out that access to safe food is currently much closer to a satisfactory level than ever before. It can be foreseen in the future, that the full exploitation of advantages offered by LAMP-based microchips will undoubtedly promote the sustainable quality and safety of food products.

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