Biosensors and Bioelectronics 135 (2019) 120–128

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Fully integrated and slidable paper-embedded plastic microdevice for point-of-care testing of multiple foodborne pathogens

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ARTICLE INFO

Keywords:
Integrated microdevice
Foodborne pathogens
DNA extraction
Loop-mediated isothermal amplification (LAMP)
Colorimetric detection

ABSTRACT

This study presents a slidable paper-embedded plastic microdevice fully integrated with DNA extraction, loop-mediated isothermal amplification (LAMP), and colorimetric detection functionalities. The developed microdevice consists of three layers that allow a sliding movement to mix the sample and reagents for DNA purification, amplification, and detection in a sequential manner. An FTA card was employed in the main chamber for DNA extraction and purification from intact bacterial cells. Subsequently, LAMP reagents and fuchsin-stored chambers were pulled toward the main chambers for DNA amplifications at 65 °C. After 30 min, the detection reagents-stored chambers were then moved to main chambers for result analysis. For the detection of LAMP amplicons, a novel colorimetric fuchsin-based method was employed. The wide applicability of the integrated microdevice was demonstrated by successfully screening three major foodborne pathogens, namely *Salmonella* spp., *Staphylococcus aureus*, and *Escherichia coli* O157:H7 in food, enabling highly sensitive detection of 3.0×10^1 CFU/sample of Gram-negative bacteria (*Salmonella* spp. and *Escherichia coli* O157:H7) and 3.0×10^2 CFU/sample of Gram-positive bacteria (*Staphylococcus aureus*) within 75 min. The portable and integrated microdevice presented in this study holds significant promise for point-of-care applications to accurately and rapidly diagnose and control diseases.

1. Introduction

Rapid, sensitive, and selective diagnosis of pathogens is an important tool in the management of infectious diseases. Recently, the idea of a sample-in-answer-out device has become highly desirable for point-of-care testing (POCT) or diagnosis in resource-limited settings. An integrated molecule microdevice based on nucleic acid amplification combined with sample purification and direct detection in a platform is a rapidly growing field for pathogen identification with numerous applications (Connelly et al., 2014; Trinh and Lee, 2017; Safavieh et al., 2014; Dhar and Lee, 2018). It helps bridge the gap between diagnostic technology in the laboratory and in the field owing to its affordability, portability, ease of use, and the ability to operate with little or no supporting equipment (Martinez et al., 2010).

For nucleic acid amplification, loop-mediated isothermal amplification (LAMP) - which is an isothermal amplification method - can outperform conventional polymerase chain reaction (PCR) by the elimination of thermal cycling control steps. The series of alternating temperatures in PCR makes it very difficult to fabricate a low-cost and portable microdevice (Notomi et al., 2015; Zhao et al., 2015; Safavieh et al., 2016; Li and Fan, 2017). The LAMP technique introduced by Notomi et al. can overcome these limitations of the conventional PCR (Notomi et al., 2000; Tomita et al., 2008). It has high sensitivity and selectivity owing to the use of more than four primers and a polymerase with high strand displacement activity to recognize 4 to 6 regions of the target gene. To extend the ability of the LAMP-based microdevice, recently, many studies attempted to integrate many functions in one platform. For example, a paper-based device that performed the two most time-consuming processes, namely sample purification and amplification, was introduced (Connelly et al., 2014). It had a multilayer structure that one linear sliding motion can enable the introduction of sample, washing buffers, amplification master mix, and detection reagents. However, it required a handheld UV lamp for endpoint analysis. Kim et al. fabricated a polyethersulfone paper-embedded biochip used for DNA amplification and detection by colorimetric reagent (Kim et al., 2018). These methods represent significant efforts in developing an integrated tool for rapid, accurate, and robust identification of pathogens. However, there are some limitations in their applications. Some systems still require the use of additional equipment such as a UV reader for detection analysis, and some still need to prepare the sample

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https://doi.org/10.1016/j.bios.2019.04.011
Received 4 March 2019; Received in revised form 3 April 2019; Accepted 5 April 2019
Available online 11 April 2019
0956-5663/

prior to application on the chip. As such, a fully integrated microdevice platform for DNA extraction, amplification, and direct detection is still urgently required for realizing a rapid method for the clinical diagnosis of disease-associated bacteria.

The first step in nucleic acid testing, sample preparation (DNA extraction and purification), is an important step that isolates DNA or RNA from the complicated biological sample and determines the template quality for the downstream analysis (Trinh et al., 2018). It is critical to eradicate the factors that may interfere with the amplification process (Zhang et al., 2013). Although many extraction methods were applied for microdevices such as the use of magnetic or silica beads or modified membrane, the extraction efficiency was not very high for biological specimens, such as whole blood, plant cells, tissue culture cells, and microorganisms. Moreover, in some cases, the sample must be treated prior to the extraction. Recently, Whatman FTA cards, a commercial product for nucleic acid extraction and purification, has been successfully employed for a POCT assay to improve the purification efficiency and facilitate high throughput (Ye et al., 2018; Lu et al., 2016; Zhang et al., 2014). Thus, an FTA card can extract and purify DNA from various biological sample types as it contains lysis buffer and protein denaturants for breaking cell membranes and damaging proteins. Moreover, the FTA card can immobilize DNA inside its matrices and keep it safe from other harmful factors (Choi et al., 2015) after the sample preparation process. Owing to these advances, the FTA card has shown great application potential for on-chip DNA extraction with high performance at low bacteria concentration in the sample.

To improve the specificity and sensitivity of POCT application, new labels including europium (III) chelate microparticles (Liang et al., 2015), fluorescent magnetic nanobeads (Huang et al., 2019), fluorescent microsphere (Chen et al., 2013) have also emerged in conventional lateral flow assay as alternative tools to assist in result read-out. However, one of the biggest limitations in POCT application of these methods is that they require UV source for result analysis, therefore reducing the portability of the device. Moreover, the use of these fluorescent labels for POCT devices can cause health risk for users due to their toxicity. Therefore, there is a need for developing a colorimetric-based microdevice which is portable, user-friendly, sensitive, and selective. Colorimetric detection methods offer analytical tools for POCT application with many advantages including fast, simple, and low-cost detection (Qin et al., 2017, 2018; Lai et al., 2017).

In terms of detection methods for LAMP amplicons, there are direct and indirect methods for analyzing the amplicons. On the one hand, some common DNA intercalators such as SYBR Green I and ethidium bromide (EtBr) are used to detect the DNA produced in the LAMP reaction directly as these dyes strongly exhibit a fluorescence signal upon binding to double-stranded DNA. Although they are sensitive and specific for endpoint analysis, their applications still require an extra instrument such as a UV machine for signal read-out, which causes some limitations such as a bulky system, high cost, a false positive signal, and the risk to users owing to the use of highly toxic dyes. Recently, we introduced a new dye called fisetin, which is a bio-compound extracted from plants, for application in the analyses of LAMP amplicons, but it still requires a UV machine for the signal read-out (Trinh and Lee, 2018). On the other hand, indirect methods that detect LAMP amplicons using pyrophosphate are commonly applied in LAMP. Pyrophosphate, which is synthesized massively in the reaction, results in turbidity with magnesium ion in the reaction solution. Thus, monitoring the turbidity is a unique way to detect LAMP amplicons (Mori et al., 2001). Furthermore, colorimetric metallochromic indicators such as hydroxyl naphthol blue (Goto et al., 2009; Seok et al., 2017) or eriochrome black T (Seo et al., 2017) were employed to indicate the production of pyrophosphate. As these methods detect the by-product rather than the main product, their applications are limited. When pyrophosphate is synthesized in a large amount, it will inhibit the activity of polymerase, and thus, decrease the efficiency of the amplification (Park et al., 2010). To overcome these shortcomings, we introduce a direct colorimetric method for detecting LAMP amplicons by using fuchsin. Fuchsin is a triphenylmethane dye and has been widely used in the industry as a textile dye and in the research field as a nucleus stain for biological samples (Graham et al., 2017; Weldu et al., 2013). Inspired by the application of fuchsin, we employed it in our study for staining DNA produced after the LAMP reaction. Hence, fuchsin can serve as an effective colorimetric method for POCT.

In this study, we fabricated a fully integrated and slidable paper-embedded microdevice allowing for the POCT of DNA from foodborne pathogens integrating DNA extraction, LAMP-based amplification, and colorimetric detection in a seamless manner. With the use of an FTA card, DNA extraction and purification were applied to collect DNA from a low-level sample and to eliminate multiple steps generally required in conventional methods. In particular, chemicals and reagents for LAMP amplification and colorimetric detection were stored in paper discs as the reagent carrier and were embedded into the microdevice for readily performing sample preparation, amplification, and detection. Therefore, different chemicals and reagents introduced for the reaction could be transferred sequentially to the main chambers to realize the amplification and detection by simply sliding the middle layer of the microdevice in a continuous manner. Thus, the sliding motion along with the ability of the paper eliminated the use of complicated instruments such as a valve or pump for serial introduction of the sample, washing buffer, amplification reagents, and detection chemical. For practical applications, the potential of the developed microdevice for the rapid detection of the three most common foodborne pathogens (*Salmonella* spp., *Escherichia coli* O157:H7 (*E. coli* O157:H7), and *Staphylococcus aureus* (*S. aureus*)) was demonstrated. Our final goal is to identify these bacteria with reliable sensitivity and specificity in a rapid and affordable manner.

2. Materials and methods

2.1. Chemicals and materials

An FTA classic card, FTA purification reagent, and bovine serum albumin (V fraction) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TE buffer (10 nM Tris-HCl, 0.1 nM EDTA, pH 8.0) was purchased from Thermo Fisher Scientific (CA, USA). Regenerated cellulose membrane filter (pore size 0.2 μm) was purchased from CHMLAB group (Barcelona, Spain). The LAMP kit, which contained *Bst* 2.0 WarmStart DNA Polymerase, 10 × isothermal amplification buffer, dNTP mix, and 100 mM $MgSO_4$, was obtained from New England BioLabs (Ipswich, MA, USA). Basic fuchsin and sodium sulfite were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (HCl) was purchased from Daejung (Korea). The DNA ladder (100 bp) was purchased from Takara and agarose powder was purchased from BioShop (Burlington, ON, Canada). Poly(methyl methacrylate) (PMMA) substrates with the thicknesses of 5 mm and 2 mm were purchased from Goodfellow (Coraopolis, USA). Mueller Hinton broth (MHB) was purchased from Becton Dickinson (Franklin Lakes, NJ, USA) and nutrient broth (NB) was purchased from Neogen (Lansing, MI, USA). Ethidium bromide dye was purchased from Dynebio (Seongnam, Korea).

2.2. Microdevice fabrication

Fig. 1a shows the overall structure of the integrated plastic microdevice composed of three layers. In brief, the PMMA cage including the top, middle, and bottom layers was created using a computer numerical control machine. Thus, the top and bottom layers served as a closed cage and assisted in the smooth sliding movement of the middle layer during the serial operation steps of the microdevice. Moreover, the main chambers were thoroughly engraved through the top and middle layers and part of the bottom layer and each chamber in the bottom layer holds an FTA card for DNA extraction. The middle layer contains three parallel lines: the main chambers and subsidiary chambers 1 and

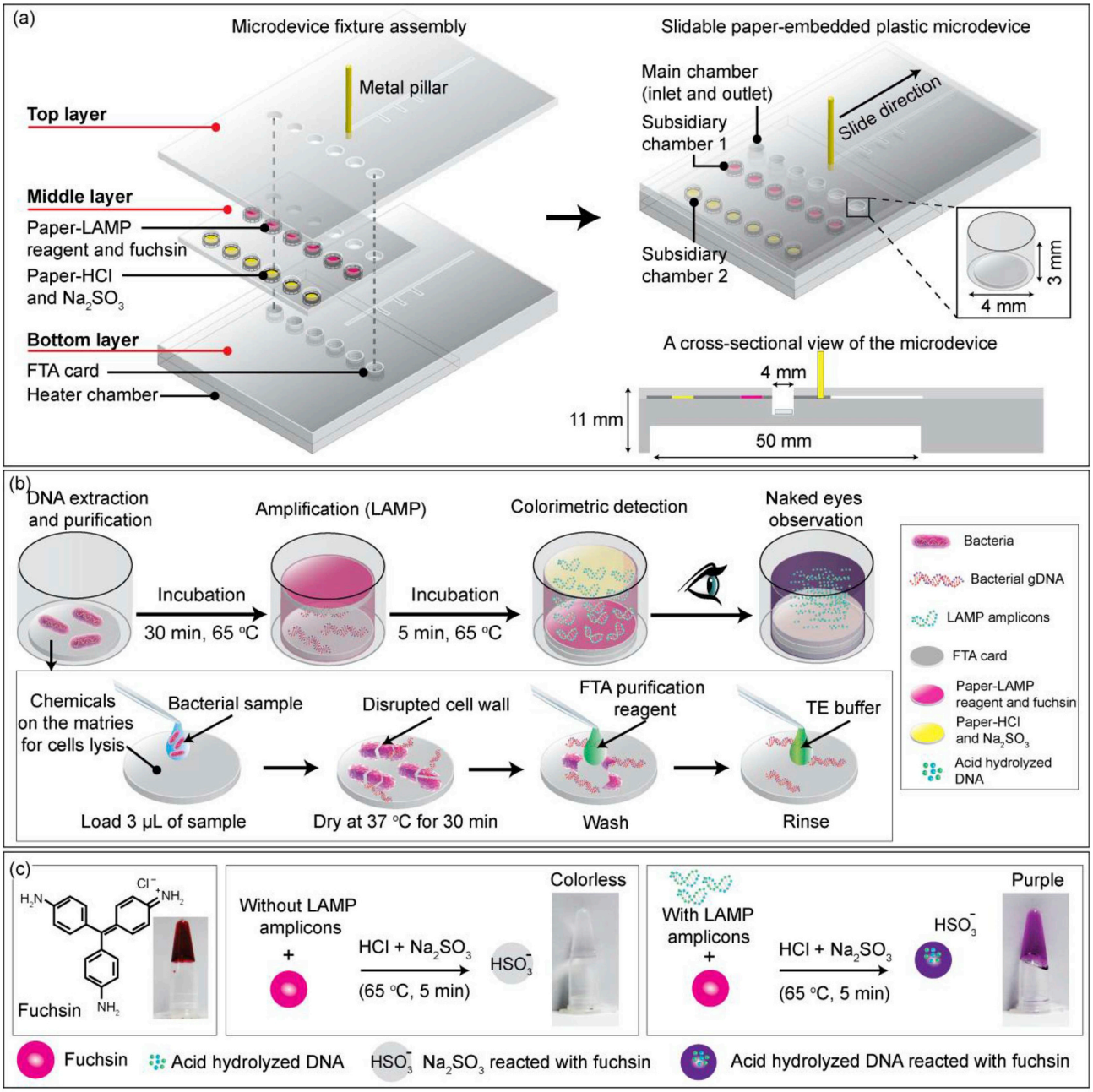

Fig. 1. (a) Illustration of the fully integrated microdevice fabricated with three layers for pathogens detection. (b) The schematic illustration of the overall experimental processes including DNA extraction/purification, amplification, and detection steps for the detection of multiple foodborne pathogens. (c) Schematic showing the colorimetric detection of LAMP amplicons using fuchsin.

2. Subsidiary chambers 1 were embedded with paper discs (pink color), which contained the mixture of LAMP reagents and fuchsin for nucleic acid amplification and subsequent colorimetric detection. Subsidiary chambers 2 were embedded with paper discs (yellow color) for storing HCl and Na_2SO_3. The bottom layer contained part of the main chambers and heater chamber where a rubber heat pad could be incorporated for realizing a portable microdevice. The functionally integrated plastic microdevice is shown in Fig. 1a. Moreover, a cross-sectional view of the microdevice precisely demonstrates the dimensions of the microdevice (1.1 cm in thickness, 8 cm in length, and 5 cm in width) and those of the main chamber (0.4 cm in diameter and 0.3 cm in height).

The overall schematic for the detection of foodborne pathogens in each main chamber is shown in Fig. 1b. With the use of an overnight bacteria culture solution, the DNA was extracted by employing a small FTA card (3 mm in diameter) containing a mixture of strong buffers and chemicals (cell lysis, protein denaturants, free radical trap) for performing DNA extraction and purification (Connelly et al., 2014). After two rounds of washing the FTA card using a washing buffer, the LAMP reaction was performed simultaneously. The mixture of LAMP reagent and fuchsin was pre-stored in the paper disc (3 mm in diameter) and dried for 1 h at the temperature assumed to be room temperature. As fuchsin is stored in the paper disc, the paper discs in subsidiary chambers 1 appeared magenta in color. For observing the LAMP amplicons with the naked eye, colorimetric detection was achieved by the color change of the paper disc and solution after performing acid hydrolysis of DNA and Na_2SO_3 treatment steps in subsidiary chambers 2. The mechanism for color change when the target exists or otherwise is explained in Fig. 1c.

For the detection of DNA, Fig. 1c shows the schematic of the colorimetric method for the detection of LAMP amplicons. No color and

dark purple color were observed in subsidiary chambers 2 for negative and positive signal amplifications, respectively. On the one hand, with the absence of LAMP amplicons, sodium sulfite bleached the solution into a colorless solution. The mechanism of this phenomenon is explained by a hypothesis that the central C atom of fuchsin molecule is attacked by the sodium sulfite molecule. This leads to the loss of the chromophoric structure of fuchsin, which becomes colorless fuchsin leucosulfonic acid (also known as leucofuchsin). On the other hand, with the presence of LAMP amplicons, the fuchsin solution is not decolorized. Under a mild acidic condition, DNA is hydrolyzed, it goes through purine cleavage and deoxyribose transformation to expose aldehyde group. The aldehyde group produced in acid hydrolysis process attacks sodium sulfite in the central C atom of fuchsin, therefore sodium sulfite cannot bind to central C atom of fuchsin. That phenomenon keeps fuchsin chromophoric structure (Mello and Vidal, 2017). These chemical reactions of the colorimetric detection method were represented in detail in Fig. S1. The color of this solution remained dark purple, representing positive signal amplification.

2.3. Microdevice operation

As shown in Fig. S2, by sliding the middle layer, the integrated microdevice allows sequential operation of DNA extraction from live bacteria together with DNA amplification using LAMP, and subsequent colorimetric detection with the naked eye. As shown in Figs. S2a and 3 μL of the overnight bacterial culture solution was introduced into the main chambers where the FTA card was subjected to DNA extraction for 30 min. For the preparation of the DNA sample for LAMP assay, the FTA card was washed with 25 μL of FTA purification reagent and TE buffer for 5 min at room temperature, separately. In this step, all the washing solutions were discarded using a pipette. Subsequently, to perform LAMP assays, the paper discs containing the mixture of LAMP reagents and fuchsin were introduced into the main chambers by sliding the middle layer one step forward as shown in Fig. S2c. Subsequently, 12.5 μL of deionized water was added into the main chambers for supplying the solution for the LAMP process. As shown in Fig. S2d, the main chambers were temporarily closed by continuously sliding the middle layer one more step forward, and they were simultaneously supplied with heat for DNA amplification at 65 °C for 30 min. Finally, colorimetric detection was realized by adding the paper discs in subsidiary chambers 2 into the main chambers and heating at 65 °C for 5 min. The color change of the LAMP solution was detected with the naked eye as well as digital images using a Nikon D7100 camera and analyzed using ImageJ analysis. The whole operation process using the fully integrated microdevice required approximately 75 min.

2.4. Bacteria culture

In this study, three kinds of foodborne pathogens including Gram-positive (*S. aureus* (KCCM 11806)) and Gram-negative (*Salmonella* spp. (ATCC 25923), *E. coli* O157:H7 (ATCC 43895)) bacteria were tested. *Salmonella* spp. was grown in both liquid culture media and agar plates. *E. coli* O157:H7 was cultured in 5 mL of Luria Bertani broth low salt (LB) medium containing 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl in 1 L of distilled water at 37 °C for 16 h by constantly shaking at 200 rpm. We selected the *eae*A target gene of *E. coli* O157:H7, a well-known factor for assessing the virulence of the target pathogen. *Salmonella* spp. was grown in 5 mL of Nutrient broth (NB) medium (1 L NB containing 3 g of beef extract and 5 g of enzymatic digest of gelatin) at 37 °C for 16 h by constantly shaking at 200 rpm. The *inv*A genes were chosen as the targets for identifying *Salmonella* spp., *S. aureus* was cultured in 5 mL of Mueller-Hinton Broth (MHB) medium containing 3 g of beef extract, 17.5 g of acid hydrolysate of casein, and 1.5 g of starch in 1 L of distilled water at 37 °C for 16 h by constantly shaking at 200 rpm. Viable counts were determined by performing serial dilution plating on solid NB agar media for the *Salmonella* spp., solid LB agar media for *E. coli* O157:H7, and MHB agar media for *S. aureus*, and incubating at 37 °C for 16 h. For indicating the applicability of FTA card on real samples, *E. coli* O157:H7 was spiked into juice and *E. coli* O157:H7, *Salmonella* spp., and *S. aureus* were spiked into milk.

2.5. Temperature control

The temperatures were measured using an infrared (IR) camera (FLIR Thermovision A320) and IR images were analyzed using an image analyzer (ThermoCAM Quick Plot). For controlling the temperature during the LAMP assays, a lab-made rubber heater consisting of four elements (circuit protector, power supply, temperature controller, and rubber heat pad) was employed and the temperature was controlled at 65 °C (Trinh and Lee, 2017). In this study, the rubber heat pad was embedded in the bottom layer of the integrated microdevice.

2.6. DNA extraction using the FTA card

The small piece of FTA card was prepared for performing DNA extraction. First, 3 μL of bacteria was applied on the FTA card using a pipette and incubated at room temperature for 30 min for DNA extraction. After drying the FTA card, various volumes of the FTA purification reagents and TE buffer (25, 50, 75, 100, 125, 150, 175, and 200 μL) were applied to wash and rinse the FTA card for optimizing both factors, respectively. Moreover, the effect of the number of washing times on the extraction efficiency was evaluated (once and twice for each solution). Furthermore, to optimize the size of the FTA card required for DNA extraction, FTA cards of various sizes (2, 2.5, 3, 3.5, 4, and 5 mm in diameter) were tested. The limit of detection (LOD) was also examined by applying various input bacteria concentrations for DNA extraction using the FTA card. For data analysis, all the DNA samples extracted by the FTA card were used to perform LAMP and these results were observed through agarose gel electrophoresis and naked-eye-based colorimetric detection methods using fuchsin as previously described.

2.7. LAMP assay

The primer sets were designed with PrimerExplorer 5 for amplifying the *eae*A gene of *E. coli* O157:H7, the *nuc* gene of *S. aureus*, and the *inv*A gene of *Salmonella* spp. as described in our previous report (Trinh et al., 2018). For an off-chip LAMP assay, the 25 μL reaction mixture contained 1 × isothermal amplification buffer, 6 mM of $MgSO_4$, 1.4 mM of dNTPs, 1.6 μM of each inner primer (FIP and BIP), 0.2 μM of each outer primer (F3 and B3), 0.8 μM of each loop primer (LF and LB), and 8 units of *Bst* 2.0 WarmStart DNA Polymerase. After mixing the LAMP reagents, the solution was transferred into the tube containing the FTA card and the reaction was carried out at 65 °C for 30 min. All the experiments required a positive reaction containing the DNA target, and a negative reaction without any target, to test this approach. For an on-chip LAMP assay, the mixture of LAMP reagents was similarly prepared and stored on the paper discs. Moreover, as the microdevice was designed to amplify and detect three types of bacteria simultaneously, each paper disc was soaked with different primer sets as described in a previous report (Trinh and Lee, 2018). For on-chip LAMP assays, the sample volume was only 12.5 μL, which was two times smaller than that used for off-chip LAMP assays.

2.8. Colorimetric detection of LAMP amplicons

To develop a simple colorimetric detection method applicable for POCT, fuchsin is newly adopted in this study. Two steps are required to realize colorimetric detection. Based on the proof of concept, the acid hydrolysis process, generally performed with an HCl solution, is the first step to hydrolyze DNA and expose the aldehyde group in the DNA structure. Thus, 5 μL of LAMP amplicons were first added to 0.5 μL of

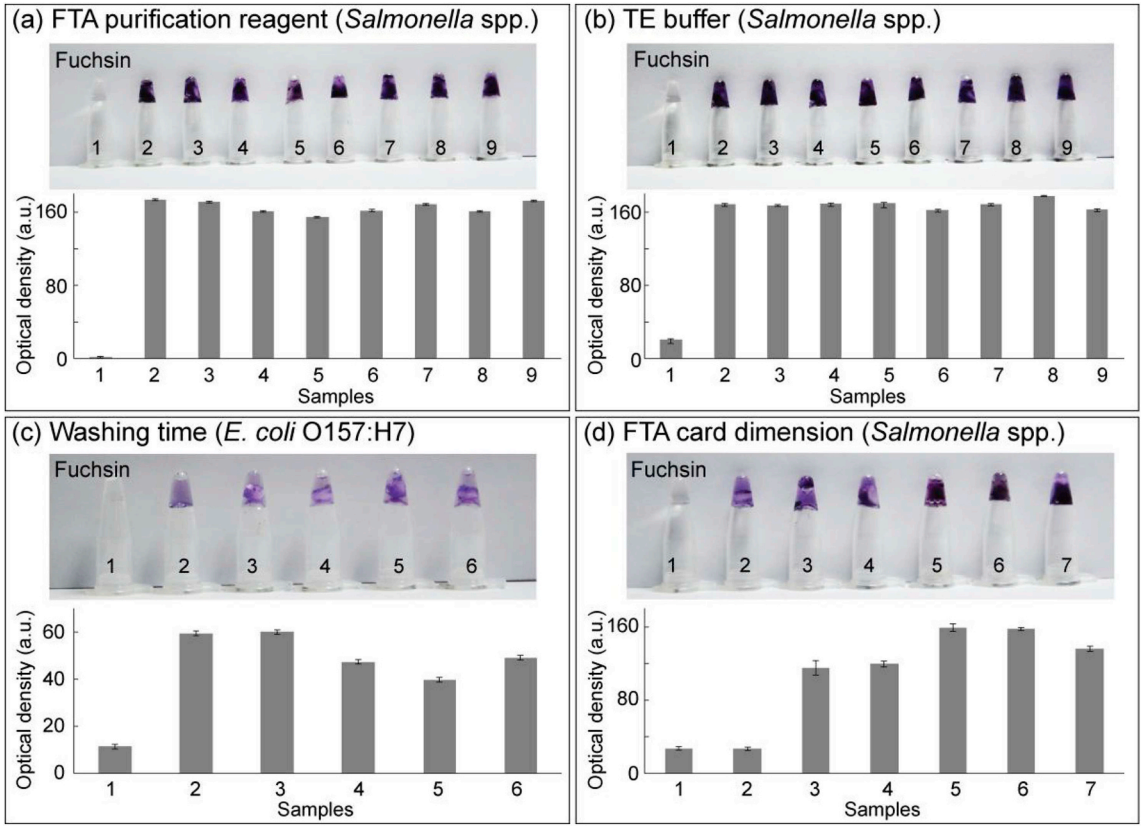

Fig. 2. Optimum washing volume of the washing solution: (a) FTA purification and (b) TE buffer: Number 1, negative control. Numbers 2–9, 25–200 μL of washing solution. (c) The effect of the optimum number of washing times on the DNA extraction efficiency: Numbers 1, 2, negative and positive control. Numbers 3, 4, one times of washing and numbers 5, 6, two times of washing. (d) The effect of various sizes of FTA cards on the DNA extraction efficiency: Number 1, negative control. Numbers 2–7, FTA cards with diameter 2, 2.5, 3, 3.5, 4, and 5 mm, respectively. All the experiments were repeated three times.

HCl (0.5 mM) and reacted at 65 °C for 5 min. Subsequently, sodium sulfite (Na_2SO_3) (51 μM) and fuchsin (9 μM) were continuously added to the tube. The condition for optimization for POCT application was also shown in Fig. S3. Afterward, the LAMP signal was observed through the color change of the solution with the naked eye as well as digital images. In the case of on-chip detection, all the reagents were embedded into the paper disc for simply realizing detection integrated with DNA extraction and amplification within a single microdevice.

3. Results and discussions

3.1. Optimization of DNA extraction from bacterial culture solution using FTA card

As shown in Fig. 2, with the use of the FTA card for DNA extraction, the DNA of *Salmonell*a spp. and *E. coli* O157:H7 were successfully isolated and amplified using LAMP and its signal was indicated using fuchsin. After being incubated with the sample, the card is washed with FTA purification reagents and TE buffer to remove the amplification inhibitors. Based on the production information of Whatman FTA cards, the FTA card (3 mm in diameter) is washed with 200 μL of FTA purification reagent and 200 μL of TE buffer and the washing steps are repeated twice in the tube. As shown in Fig. 2a and b, the LAMP amplicons of *Salmonell*a spp. were analyzed colorimetrically using fuchsin with samples 2–9 of the image corresponding to 25–200 μL of the volume of each washing buffer (FTA purification reagent and TE buffer). The washing procedure was performed twice and the sample 1 is the negative control for LAMP. Moreover, there was no significant difference in optical density among the samples. From these results, 25 μL of the FTA purification reagent and 25 μL of TE buffer were chosen to wash the FTA card after the cell lysis. In other words, the FTA card could be fully cleaned when the concentration of the washing buffers (FTA purification reagent and TE buffer) was nearly 10 times lower than that used in the manufacturer's protocol.

Concerning the number of washing times, the FTA cards used for LAMP amplification contained the same concentration of bacteria as well as the optimum volume of washing buffers in previous experiments. As shown in Fig. 2c, the LAMP amplicons of *E. coli* O157:H7 were also analyzed using the colorimetric detection method. Samples 1 and 2 were negative and positive controls, respectively. Two different washing times were evaluated and demonstrated in samples 3–6. Samples 3, 4 and samples 5, 6 demonstrated the results obtained by carrying out the washing step once and twice, respectively. Based on the color analysis of LAMP amplicons using ImageJ, there was a relatively similar color intensity in optical density in the graph. Therefore, washing once could still effectively purify DNA from impurities. Based on these results, we can conclude that the FTA card could be readily employed in the integrated microdevice.

For optimizing the size of the FTA card, FTA cards of various sizes were tested to perform DNA extraction (Fig. 2d), and 3 μL of bacteria was used for application on a single FTA card. As shown in the real image, the LAMP amplicons of *Salmonell*a spp. were analyzed through colorimetric detection with samples 2–7 corresponding to the FTA card with the diameters of 2, 2.5, 3, 3.5, 4, and 5 mm, respectively. The sample 1 was a negative control. The color of the LAMP amplicons increased with the increase in the size of the FTA card (from 2.5 to 4 mm) except for the FTA card of diameter 5 mm. A larger FTA card generally requires a larger volume of washing buffers. The LAMP efficiency could be reduced when the inhibitors were not completely washed away from the FTA card. From these results, the FTA card 2.5 mm

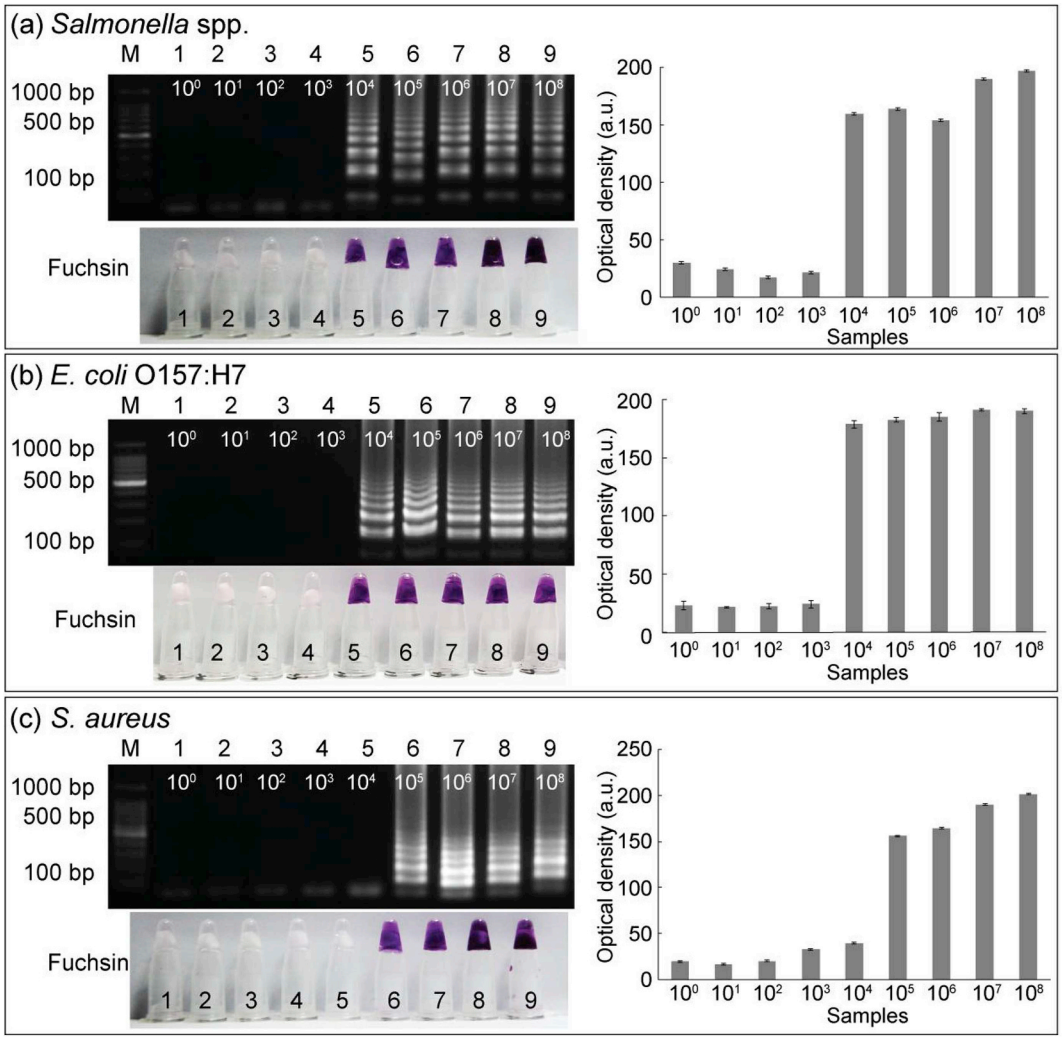

Fig. 3. (a) Various input concentrations (eight-fold serial dilutions) of *Salmonella* spp. extracted using the FTA card. (b) Various input concentrations (eight-fold serial dilutions) of *E. coli* O157:H7 extracted using the FTA card. (c) Various input concentrations (eight-fold serial dilutions) of *S. aureus* extracted using the FTA card. All the experiments were repeated three times.

in diameter was chosen for on-chip DNA extraction.

3.2. Limit of detection of bacteria using FTA card for DNA extraction

To determine the LOD, two Gram-negative bacteria, *Salmonella* spp. and *E. coli* O157:H7, and one Gram-positive bacteria, *S. aureus*, were examined. For bacteria detection, several factors such as DNA extraction efficiency, LAMP amplification, and visual colorimetric detection could affect the LOD. However, the efficiency of DNA extraction using a low concentration of bacteria is the critical factor affecting the LOD when using an integrated microdevice. Fig. 3a shows the results of LAMP amplicons from *Salmonell*a spp., which were analyzed through agarose gel electrophoresis with lanes 1–9 of the gel corresponding to 10^0–10^8 CFU/mL of the initial bacteria concentration, respectively. The DNAs of the target bacteria with the concentration ranging from 10^4–10^8 CFU/mL were successfully amplified. Owing to the use of 3 μL of bacteria sample for each reaction, the LOD was approximately 3×10^1 CFU/sample. Moreover, a visible color of the LAMP amplicons in the real image as well as optical density in the graph could be observed using fuchsin-based colorimetric detection. Similarly, Fig. 3b also showed that the lowest amount of *E. coli* O157:H7 that could be detected was approximately 3×10^1 CFU/sample. Fig. 3c shows the results of LAMP amplicons from *S. aureus*, which were analyzed through agarose gel electrophoresis with lanes 1–9 of the gel corresponding to 10^0–10^8 CFU/mL of bacteria concentration, respectively. The DNAs of the target bacteria with the concentration ranging from 10^5–10^8 CFU/mL were successfully amplified. In addition, a visible color of the LAMP amplicons in the real image as well as optical density was shown in the graph. Owing to the use of 3 μL of bacteria sample for each reaction, the LOD was approximately 3.0×10^2 CFU/sample. From these results, it could be concluded that it was likely difficult to extract DNA from Gram-positive bacteria (*S. aureus*) as compared with Gram-negative bacteria (*Salmonell*a spp. and *E. coli* O157:H7) by using the FTA card. This phenomenon can be obtained due to the difference in cell wall structure between Gram-positive and Gram-negative bacteria. Gram-positive bacteria is surrounded by a thick layer of peptidoglycan, which prevents Gram-positive bacteria cell from harsh environment. Moreover, through the Gram-positive peptidoglycan layer, there are many other components such as teichoic acid, polysaccharide, and protein. These factors build a stable cell wall to protect the bacteria cell. However, in Gram-negative bacteria, the cell wall structure is thinner and simpler. Therefore, methods used for genome extraction are more effective in Gram-negative (Silhavy et al., 2010).

3.3. Effect of real samples on DNA extraction, LAMP amplification, and colorimetric detection

To indicate the effect of real samples on the performance of the

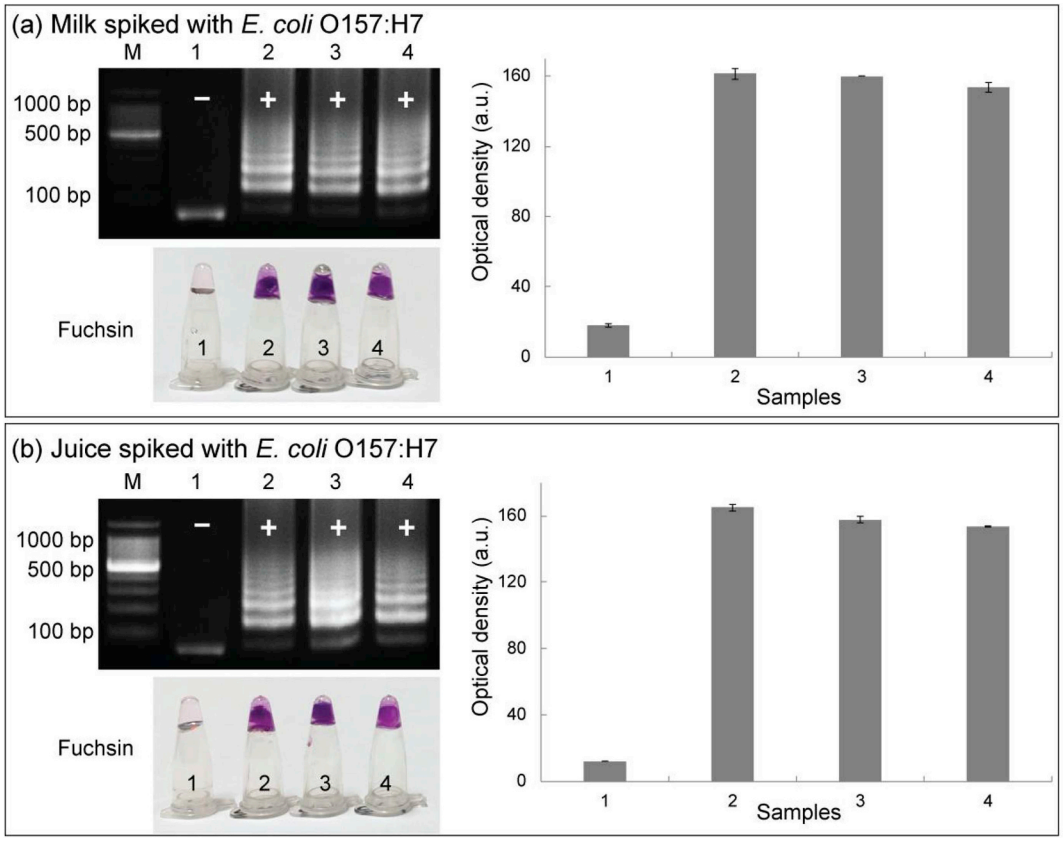

Fig. 4. Effect of real samples on DNA extraction, LAMP amplification, and colorimetric detection. (a) Milk spiked with *E. coli* O157:H7. (b) Juice spiked with *E. coli* O157:H7. Lane M: 100 bp ladder. Lane 1: negative control. Lanes 2–4: amplified results from real samples with repeated experiments.

microdevice, *E. coli* O157:H7 (10^4 CFU/mL) was spiked into milk and juice. *E. coli* O157:H7 genome from each sample was extracted and incubated using FTA card (Fig. 4). Chemicals impregnated inside FTA card matrices lysed bacterial cell membranes and denatured other cell proteins. DNA was released, captured in the matrice and protected from oxidation, nuclease, and contamination. In washing step, buffer washed away inhibition components in milk and juice. This process eliminated enzyme inhibitors in crude sample and resulted in purified DNA for further amplification and colorimetric detection. LAMP technique amplified the target gene and the colorimetric signal was achieved using fuchsin-based method. The results in Fig. 4 indicated that our method is capable of detecting pathogens such as *E. coli* O157:H7 in real samples. Moreover, the feasibility of the microdevice for detecting other bacteria in milk and the specificity were shown in Figs. S4 and S5, respectively.

3.4. Fully integrated operation for the detection of multiplex pathogens using the slidable microdevice

Real image of the microdevice system for rapid identification of foodborne pathogens was shown in Fig. S6. It included the sliding paper-embedded plastic microdevice and a heat supply. To obtain DNA from the intact bacteria sample, we used the FTA card embedded in the chambers of the microdevice for extraction. For DNA amplification and detection, paper discs were employed to store the reaction reagents. The introduction of sample between extraction, amplification, and detection zones was controlled by a sliding movement. Fig. 5a shows the photos of the sequential operation process using the integrated microdevice. In addition, the cross-sections of the microdevice are distinctly shown, demonstrating the location of the paper discs during the microdevice operation. Specially, the whole operation process was further shown in the supplementary movie. First, a spiked milk was injected into the main chamber, which contained the FTA card. The sample pretreatment step was carried out here and purified DNA was immobilized in the FTA card. By simply sliding the middle layer of the microdevice, the extracted DNA obtained from the FTA card was mixed with the LAMP reagent and fuchsin. The solution inside the main chambers was incubated for DNA amplification at 65 °C for 30 min which was selected as the optimum reaction time for performing on-chip LAMP amplification (Fig. S7). Fig. S8 shows the real image of the bottom layer incorporated with the rubber heat pad. To realize the portable microdevice, the rubber heat pad was embedded on the lower part of the bottom layer of the integrated microdevice. Thus, the main chambers can be located in the center area of the heater for homogeneous temperature distribution along all six main chambers. Before applying spiked milk into the microdevice, cultured solution was employed as sample for bacteria detection using the fully integrated microdevice (Fig. S9). As shown in Fig. 5b–d, three types of foodborne pathogens were successfully identified by using the microdevice. As the results, the success of extraction and amplification was indicated by agarose gel electrophoresis results (Fig. 5b). To perform on-chip detection, the chip was then slid to align the main chambers with the detection port for signal read-out as shown in Fig. 5c. Amplified DNA was acid hydrolyzed under hot acid condition to expose the aldehyde group. The presence of acid-hydrolyzed DNA protected the color of fuchsin from the bleaching activity of sodium sulfite in the solution, which resulted in the purple color of the chambers. In contrast, chambers without amplified DNA turned colorless and exhibited white color. ImageJ was also used to analyze the color change of each chamber before and after the reaction. As shown in Fig. 5d, the relative color intensities of the main chambers were almost similar before the LAMP reaction; however, after the LAMP reaction, there was a significant decrease in the color intensity in the negative chambers

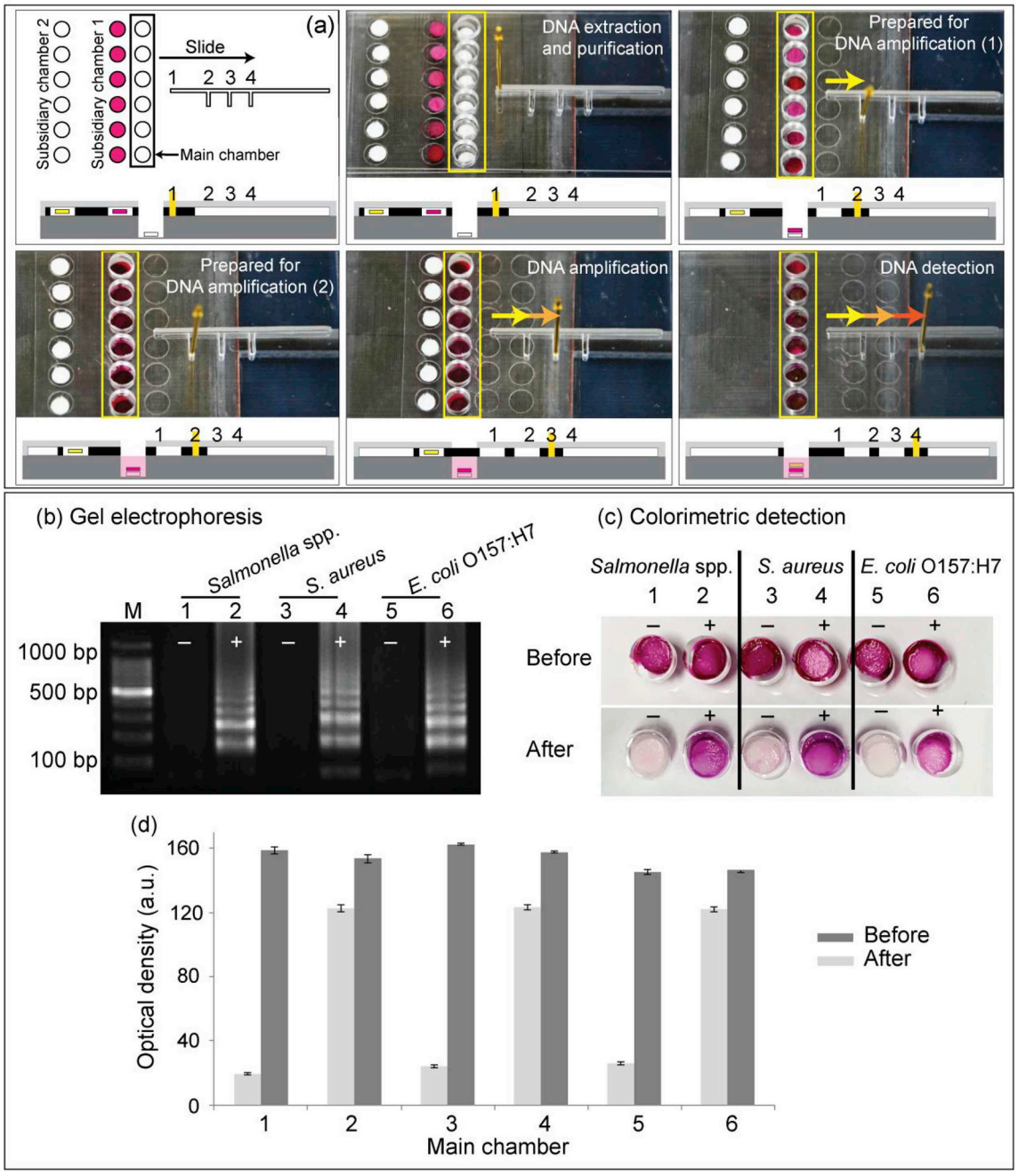

Fig. 5. (a) Photographs show the operation for performing integrated processes of extraction, amplification, and detection using a slidable microdevice. (b) Results of agarose gel electrophoresis obtained from milk. (c) Results using fuchsin-based colorimetric detection. (d) The graph showing the optical density analyzed inside the main chambers. All the experiments were repeated three times.

whereas the positive chambers remained purple. This result proved that our strategy was effective in discriminating a negative result from a positive one simply based on the color change. Using this integrated microdevice, the screening of a target from a mixture of raw samples was effectively achieved in a fast and facile manner.

4. Conclusions

In this work, we developed a fully integrated, slidable, and reusable plastic microdevice embedded with paper discs for naked-eye pathogen detection. The microdevice performed all key steps in a nucleic acid-based test including sample purification using FTA card, amplification using LAMP technique, and fuchsin-based colorimetric detection. Multiple foodborne pathogens of both Gram-negative and Gram-positive origins were successfully identified with high sensitivity and specificity even when real samples were used. We believe that the introduced microdevice will pave the way for early diagnosis of infectious diseases, and can extend its wide usage for real-time monitoring for public healthcare and biosafety when a more compact heater is installed.

CRediT authorship contribution statement

Kieu The Loan Trinh: Conceptualization, Investigation, Writing - original draft, Visualization. **Thi Ngoc Diep Trinh:** Formal analysis, Investigation, Writing - review & editing, Visualization. **Nae Yoon Lee:** Methodology, Supervision.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No. NRF-2017R1A2B4008179). This research was also supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI18C2096).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2019.04.011.

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