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A portable viable *Salmonella* detection device based on microfluidic chip and recombinase aided amplification

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ABSTRACT

Screening of foodborne pathogens is important to prevent contaminated foods from their supply chains. In this study, a portable detection device was developed for rapid, sensitive and simple detection of viable *Salmonella* using a finger-actuated microfluidic chip and an improved recombinase aided amplification (RAA) assay. Improved propidium monoazide (PMAxx) was combined with RAA to enable this device to distinguish viable bacteria from dead ones. The modification of PMAxx into dead bacteria, the magnetic extraction of nucleic acids from viable bacteria and the RAA detection of extracted nucleic acids were performed using the microfluidic chip on its supporting device by finger press-release operations. The fluorescent signal resulting from RAA amplification of the nucleic acids was collected using a USB camera and analyzed using a self-developed smartphone App to quantitatively determine the bacterial concentration. This device could detect *Salmonella typhimurium* in spiked chicken meats from 1.3×10^2 CFU/mL to 1.3×10^7 CFU/mL in 2 h with a lower detection limit of 130 CFU/mL, and has shown its potential for on-site detection of foodborne pathogens.

Foodborne diseases are recognized as one of the most serious public health problems [1–3], which mainly result from contamination of foods by pathogenic bacteria at each link of food supply chains, such as production, processing, transportation and sales. Screening of foodborne pathogenic bacteria is a key to prevent the contaminated foods from entering the supply chains. Current bacterial detection methods mainly include culture, ELISA and PCR [4–8]. However, these methods either are either time-consuming, or lack sensitivity, or require complex pretreatment. More importantly, they rely on well-trained technicians and specialized laboratories, and cannot meet the ASSURED (Affordable, Sensitive, Specific, User-friendly, Robust, Rapid, Equipment-free and Deliverable to end-users) requirement on bacterial detection recommended by the World Health Organization [9]. Therefore, the development of simple, rapid and sensitive detection methods for foodborne pathogens is of great significance to ensure food safety.

In the past decade, various microfluidic chips, such as centrifugal [10–13], sliding [14] and finger-actuated [15–17], have been often reported for detection of pathogenic bacteria. Among them, finger-actuated microfluidic chips have attracted much attention due to their easy-to-operate and free-of-equipment advantages since simple finger press-release operations could realize the functions of pump and valve to conduct the whole bacterial detection procedures on a single chip, including loading, mixing, separation and detection. Besides, some isothermal amplification assays, such as recombinase aided amplification (RAA), loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA) and rolling circle amplification (RCA), have been often used with microfluidic chips for bacterial detection because they did not need to change temperatures for nucleic acid amplification. However, these assays could not distinguish dead bacteria from viable ones and were often subject to overestimate the number of pathogenic bacteria due to possible presence of dead bacteria in foods. To avoid this overestimation, dyes, such as propidium monoazide (PMA) or ethidium onoazide (EMA), were covalently bound with nucleic acids in the dead bacteria cells through their damaged membranes to inhibit LAMP or PCR amplification of these nucleic acids [18–20]. Recently, improved propidium monoazide (PMAxx) with better ability to distinguish viable bacteria from

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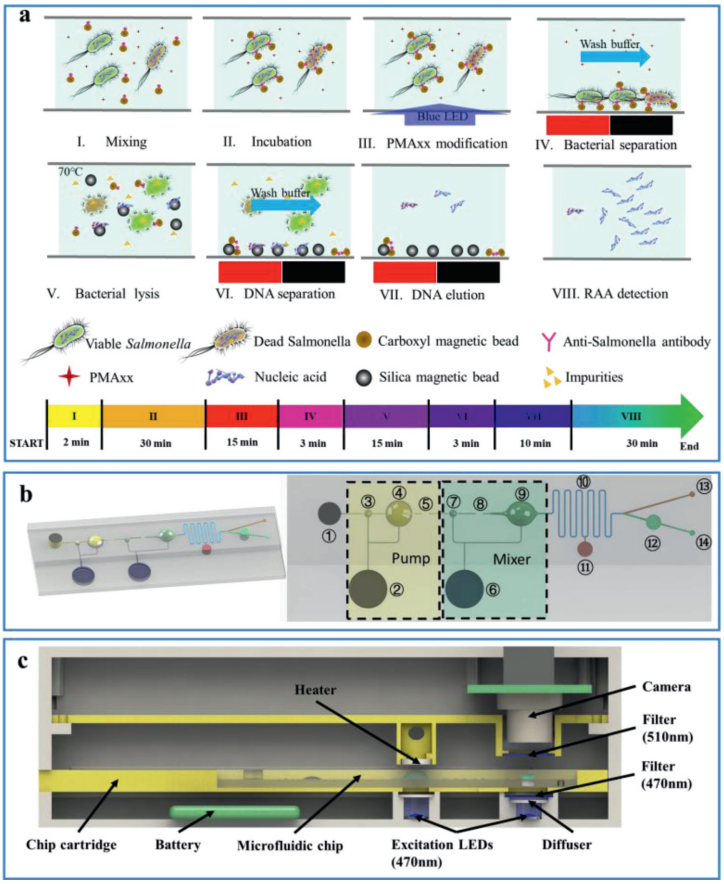

Fig. 1. (a) The procedure of bacterial detection using this *Salmonella* detection device. (b) The structure of the finger-actuated microfluidic chip (1: Inlet, 2: Pump button, 3: Pump pneumatic valve, 4: Pumping chamber, 5: Pump check valve, 6: Mixer button, 7: Mixer pneumatic valve, 8: Mixer check valve, 9: Mixing chamber, 10: Separation channel, 11: Eluent chamber, 12: RAA detection chamber, 13: Outlet 1, 14: Outlet 2). (c) The structure of this *Salmonella* detection device.

dead ones was reported to combine with quantitative real-time PCR for detection of viable *Escherichia coli* [19], *Brochothrix thermosphacta* [21] and *Staphylococcus aureus* [22].

In this study, we developed a portable detection device using a finger-actuated microfluidic chip and a PMAxx-improved real-time RAA assay for fast, sensitive and on-site detection of viable *Salmonella* typhimurium. As shown in Fig. 1, the PMAxx dye was used with the RAA assay to quantitatively detect the viable bacteria, and a finger-actuated microfluidic chip and its supporting device were developed and used with a smartphone to perform the PMAxx modification, magnetic extraction and RAA detection. A smartphone App was developed and installed to collect and analyze the fluorescent signal due to the RAA amplification of nucleic acids from viable bacteria for determination of the bacterial concentration.

As shown in Fig. 1b, the microfluidic chip contains a finger-actuated pump, a finger-actuated mixer, a serpentine magnetic separation channel, an eluent chamber and a RAA detection chamber. As shown in Fig. S1 (Supporting information), the chip is mainly composed of three PDMS layers: the top layer with a fluidic channel, the middle layer (a thin membrane with the thickness of 100 μm), and the bottom layer with a pneumatic channel. As shown in Fig. S2 and Video S1 (Supporting information), when the pump button or mixer button is pressed/released, the pressure difference between the pneumatic channel, the fluid channel and the atmospheric environment is changed, resulting in the deformation/recovery of the membrane between the pneumatic channel and the fluid channel. In this way, the sample or reagents could be easily controlled to achieve the PMAxx modification, magnetic extraction and RAA detection, when the chip was mounted on a chip cartridge and inserted into its supporting device. More details could be found in Supporting information. The top and bottom layers of the chip were made by PDMS curing. Their molds were designed using the SolidWorks software and printed by a 3D printer (Objet30 Pro, Stratasys, Holtzman St. Science Park, Israel) with photosensitive resin material. After the molds were dried, the mixture of PDMS and curing agent (10:1) was poured into the molds and cured at 60 °C for 8 h. Finally, both sides of the middle thin PDMS layer were bonded with the top and bottom layers through surface plasmon to fabricate the microfluidic chip.

To perform the PMAxx modification, magnetic extraction and RAA detection in the microfluidic chip, its portable supporting device was developed. As shown in Fig. 1c and Fig. S3 (Supporting information), this device contains a heater for the nucleic acid extraction, two excitation LEDs (470 nm) for the PMAxx modification and the fluorescent probe excitation, two narrow-band filters for the light filtering, a high-resolution camera and a smartphone for the fluorescent signal collection and analysis, and a 3D-printed holder with the chip cartridge. After the excitation light passed through the diffuser and was filtered by the narrow-band filter (465–475 nm), the green fluorescent probes resulting from RAA detection were irradiated and excited to emit the fluorescent signal with the wavelength of 520 nm, which was filtered by another narrow-band filter (515–525 nm) and collected by the camera connected to the smartphone *via* USB. The images were taken every 10 s using the self-developed smartphone App and processed using the hue-saturation-value (HSV) color model. The value was selected as the index to evaluate the fluorescent intensity, and the

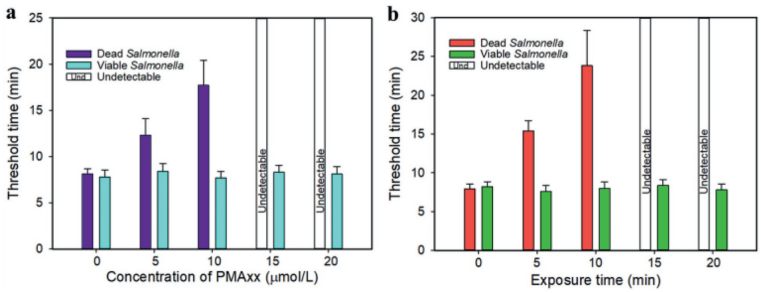

Fig. 2. (a) Optimization of the PMAxx concentration ($n = 3$). (b) Optimization of the PMAxx exposure time ($n = 3$).

time for the fluorescent intensity to reach the threshold was used to determine the bacterial concentration. According to a previous study [23], the threshold was calculated as 0.71 by adding the average value of the baseline in the amplification curve and ten times of the standard deviation.

The whole bacterial detection procedure using this device included three steps: PMAxx modification, magnetic extraction and RAA detection. Prior to test, Outlet 2 was taped on to prevent any solution from dissolving the prestored lyophilized RAA reagent. As shown in Fig. 1a, the sample, immunomagnetic beads (MNBs) and PMAxx were finger-actuated pumped into the chip and finger-actuated mixed for 25 times (I), followed by incubation for 30 min (II) under the excitation of the blue LED to allow the forming of MNB-bacteria (both dead and viable) complexes and the modification of PMAxx into the dead bacteria (III). Then, these complexes were magnetically captured at the separation channel using a NdFeB magnet (size: 20 mm × 10 mm × 5 mm, K&J Magnetics, Pipersville, PA, USA) and washed to remove the background and free PMAxx (IV). After the magnet was removed, the lysate containing protease K and silica magnetic beads (SMBs) was used to resuspend these complexes, followed by finger-actuated mixing for 25 times and incubation for 15 min at 70 °C to lyse the bacteria and release their nucleic acids from the viable bacteria, which were electrostatically adsorbed onto the surface of the SMBs to form SMB-DNA complexes (V). The SMB-DNA complexes were finger-actuated moved and magnetically captured at the separation channel and washed to remove the impurities (VI). After the pre-vacuumed bottom pneumatic chamber beneath the top eluent chamber was punctured to recover its middle membrane, the eluent was expelled from the eluent chamber into the separation channel, resulting in the elution of the nucleic acids from the SMB-DNA complexes (VII). Finally, the tap of Outlet 2 was pulled off and Outlet 1 was taped on, followed by finger-actuated moving the nucleic acids to resuspend the lyophilized RAA reagent for DNA amplification (VIII), collecting the fluorescent signal by the camera, and analyzing the data with the smartphone App to determine the bacterial concentration. More details on the procedure could be found in Supporting information.

The concentration and exposure time of the PMAxx dye have great impact on the bacterial detection and were optimized in this study. The experimental details could be found in Supporting information. The results for optimization of PMAxx concentration were shown in Fig. 2a. For dead bacteria, when the concentration of PMAxx changes from 0 μmol/L to 10 μmol/L, the threshold time for RAA detection increases from 8.1 min to 17.7 min, indicating more nucleic acids were inhibited, and when the concentration of PMAxx further increases to 15 μmol/L, no fluorescent signal is detected, indicating that all the nucleic acids were inhibited. For viable bacteria, when the concentration changes from 0 μmol/L to 15 μmol/L, the threshold time remains the same level, indicating the PMAxx concentration did not have any impact on RAA detection of viable bacteria. The results for optimization of exposure time were shown in Fig. 2b. For dead bacteria, when the exposure time changes from 0 min to 10 min, the threshold time increases from 7.9 min to 23.8 min. When the exposure time increases to 15 min, no fluorescent signal is detected, indicating all the nucleic acids were inhibited. For viable bacteria, when the exposure time changes from 0 min to 15 min, the threshold time remains the same level, indicating that the exposure time did not have obvious impact on RAA detection of viable bacteria.

The finger-actuated mixer is the key to this microfluidic chip, because it has great impact on the immune and catalytic reactions. To obtain a high mixing efficiency, the mixing chambers with different shapes (diamond, cylindrical and semi-spherical) were compared, since the cylindrical shape was commonly used and the diamond shape was recently reported [24]. The chambers were designed using Solidworks software and simulated using COMSOL software, respectively. The COMSOL simulation results were shown in Fig. 3a. When the mixing button is pressed and released, two obvious vortices are formed in the whole semi-spherical chamber, however only small vortices are formed in the partial cylindrical and diamond chambers, indicating that the semi-spherical shape has a better mixing effect than the cylindrical and diamond ones. Besides, the red dye was used to verify this simulation, and the experimental results were consistent with the simulation ones. To further interpret these results, the mixing efficiency was calculated according to the previously reported method [25]. Briefly, each collected image was grayed to obtain the gray value of each pixel, followed by calculating the standard deviation of all the gray values as the index for evaluating the mixing efficiency. As shown in Fig. 3b and Fig. S4 (Supporting information), when the mixing button is pressed and released for the same times, the mixing efficiency of the semi-spherical shape is obviously higher than those of the cylindrical and diamond ones. Thus, the semi-spherical shape was selected for the mixing chamber. Furthermore, to evaluate the actual effect of the finger-actuated mixer on nucleic acid extraction, the nucleic acids were extracted from the *Salmonella typhimurium* cells at 1.7×10^7 CFU/mL using this microfluidic chip with semi-spherical mixer and the commercial DNA extraction kit (Tiangen, Beijing, China), and the concentration of the extracted nucleic acids was measured using the ultra-micro spectrophotometer (Nanodrop 2000, Thermo Fisher, Waltham, Massachusetts, USA). As shown in Fig. 3c, when the mixing button is pressed and released from 10 times to 25 times, the concentration of the extracted nucleic acids increases from 7.4 ng/μL to 18.4 ng/μL. However, the further increase on the press-release time to 30 only results in 2.3% increase on the concentration. Thus, the optimal press-release time of 25 was used in this study.

According to the manufacture's protocol for RAA kit, 20 μL of the eluent is suggested for eluting the nucleic acids from the SMB-DNA complexes. Considering possible loss of the eluent in the transportation from the inlet to the separation channel, it may be a good choice to pre-store the eluent near the separation channel in the chip. As shown in Fig. S5a (Supporting information),

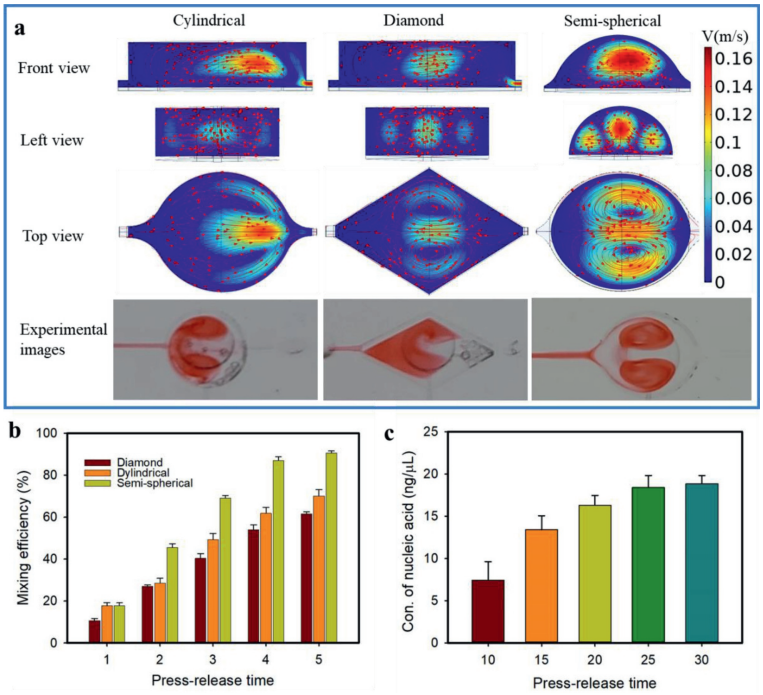

Fig. 3. (a) The simulation on three mixers with different shapes. (b) The mixing efficiency of three mixers with different press-release times. (c) The concentration of extracted nucleic acids with different press-release times.

the eluent was prestored in the top elution chamber and its bottom pneumatic chamber was sealed and vacuumed to enable the deformation of the middle membrane, resulting in the storage of more eluent, *i.e.*, the total volume of eluent was more than that of the top elution chamber. At the elution step, the bottom pneumatic chamber was punctured to allow the recovery of the middle membrane, resulting in the expelling of partial eluent into the separation channel to elute the nucleic acids. To optimize the volume of the top eluent chamber, different diameters (2–5 mm) of the bottom pneumatic chamber with different vacuum degrees (3–12 psi) were fabricated and compared. As shown in Fig. S5b (Supporting information), the volume of the red dye for 1 mm length is equal to 0.5 μL, and the length of the red dye is measured to calculate the volume of the expelled eluent after the bottom chamber was punctured. As shown in Fig. S5c (Supporting information), when the diameter increases from 2 mm to 5 mm and the vacuum degree increases from 3 psi to 12 psi, the volume of expelled red dye increases from 2.3 μL to 56.4 μL. Since more volume of eluent is subject to decrease the sensitivity, the diameter of 4 mm and the vacuum degree of 9 psi were selected to expel ~22.8 μL of the eluent into the separation channel. To verify this, a test chip was fabricated and tested for 8 times and the results were shown in Fig. S5d (Supporting information) that the mean volume is 22.9 μL with a coefficient of variation of 3.61%, indicating that a stable volume of the eluent was obtained for DNA elution.

To evaluate the performance of this microfluidic chip on nucleic acid extraction, the nucleic acids were extracted from different concentrations of *Salmonella typhimurium* using this chip. The purity of the extracted nucleic acids was evaluated using the conventional agarose gel electrophoresis. The amount of the extracted nucleic acids was determined using a commercial PCR kit (Huirui Bio, Zhuhai, China) on real-time PCR instrument (CFX96, BIORAD, Hercules, CA, USA). The result of the agarose gel electrophoresis was shown in Fig. S6a (Supporting information) that the extracted nucleic acids had a high purity, and the results of the PCR detection were shown in Fig. S6b (Supporting information) that the amount of the extracted nucleic acids was consistent with the concentration of target bacteria.

To detect the bacterial samples with unknown concentrations, *Salmonella typhimurium* with the concentrations from 1.3×10^1 CFU/mL to 1.3×10^7 CFU/mL were detected using this detection device to establish the calibration model. As shown in Figs. 4a and b and Video S2 (Supporting information), when the bacterial concentration decreases from 1.3×10^7 CFU/mL to 1.3×10^2 CFU/mL, the threshold time increases from 3.23 min to 18.47 min, and when the bacterial concentration decreases to 1.3×10^1 CFU/mL, no fluorescent signal is detected. A linear relationship between the threshold time (T) and the bacterial concentration (C) from 1.3×10^2 CFU/mL to 1.3×10^7 CFU/mL was obtained as $T = -2.99\lg(C) + 20.33$ ($R^2 = 0.98$), and the lower detection limit of this device was 1.3×10^2 CFU/mL. Compared to some recent reports for bacterial detection, this device has shown either a comparable detection sensitivity, or a shorter detection time (Table S1 in Supporting information). This might be attributed to: (1) The effective mixing and immunoreaction in the semi-spherical-shape chamber, resulting in efficient separation and concentration of target bacteria from sample background, (2) the effective extraction of nucleic acids from the viable target bacteria, resulting in sensitive RAA detection of target bacteria, and (3) the precise control of sample, reagents and temperature, resulting in good consistence of parallel detection. More importantly, this device could distinguish the viable target bacteria from the dead ones without complex operations and expensive instrumentation.

The specificity of this device was also evaluated by detecting three common foodborne pathogens, including Listeria monocytogenes, *Escherichia coli* O157:H7 and *Staphylococcus aureus* at the concentration of 1.1×10^5 CFU/mL. As shown in Fig. 4c, no fluorescent signal was detected for the three non-target bacteria, indicating this device has a good specificity due to the use of specific antibodies against *Salmonella typhimurium* for immunomagnetic separation of target bacteria and the use of specific primers for RAA detection of extracted nucleic acids.

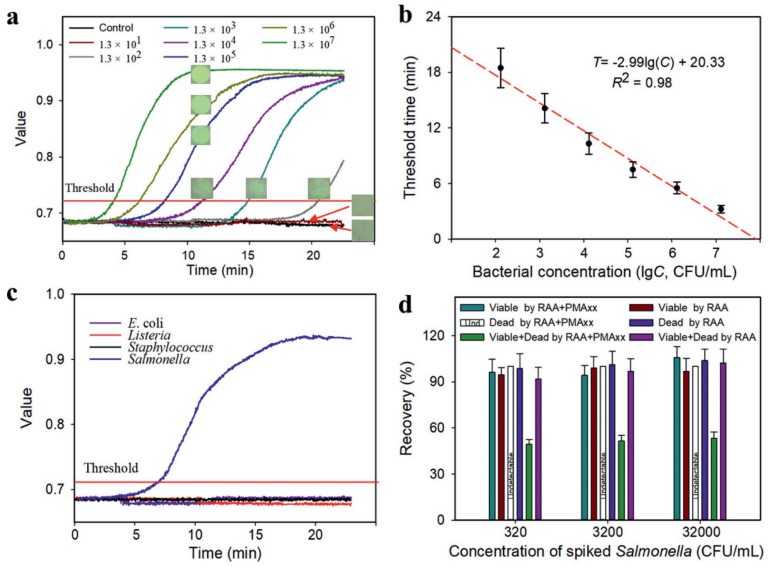

Fig. 4. (a) The RAA detection of different concentrations of *Salmonella typhimurium* with PMAxx modification. (b) The linear relationship between the threshold time and the bacterial concentration ($n = 3$). (c) The specificity of this detection device. (d) The applicability of this device for detection of viable and dead *Salmonella typhimurium* in spiked chicken meats ($n = 3$).

To further evaluate the applicability of this device for detecting *Salmonella typhimurium* in real food samples, three parallel experiments were carried out using chicken meats spiked with different concentrations of target bacteria. The chicken meats purchased from local market were pretreated according to China's food safety national standards (GB 4789.4–2016). Briefly, 25 g of chicken meats were added to 225 ml of sterilized PBS and then homogenized for 4 min using a homogenizer. After standing for 10 min to collect the supernatant, the viable and dead *Salmonella* cells with different concentrations (3.2×10^2–3.2×10^4 CFU/mL) were added into the supernatant to obtain the spiked samples containing only viable bacteria, only dead bacteria, and both viable and dead bacteria, respectively. The recovery was calculated by dividing the added concentration into the detected one to evaluate the performance. As shown in Fig. 4d, when the PMAxx modification is applied, the recovery of viable bacteria ranges from 94.3% to 105.6%; however, the recovery of dead bacteria is 0, indicating that PMAxx could effectively inhibit the amplification of the nucleic acids in dead bacteria. When the PMAxx modification is not applied, the nucleic acids from both viable and dead bacteria could be amplified and the recovery ranges from 94.6% to 98.9% for viable bacteria and from 98.5% to 103.8% for dead ones, respectively. Furthermore, viable bacteria and dead bacteria were mixed in the ratio of 1:1 and detected using the device. When the PMAxx modification is not applied, the recovery ranges from 91.8% to 102.2%. When the PMAxx modification is applied, the recovery reduces to 49.3%−53.1% due to the inhibition of nucleic acids in dead bacteria.

In conclusion, a portable bacterial detection device was successfully developed for fast, simple and sensitive detection of viable *Salmonella typhimurium*. The combination of this finger-actuated microfluidic chip and its supporting device with the RAA assay was demonstrated with efficient bacterial separation, high detection sensitivity and short detection time. The PMAxx dye was confirmed with effective inhibition of nucleic acids in dead *Salmonella* cells. Besides, the value in the hue-saturation-value color model was verified with sensitive analysis of the fluorescent signals. The experimental results showed that this detection device could quantitatively detect *S. typhimurium* ranging from 1.3×10^2 CFU/mL to 1.3×10^7 CFU/mL in 2 h with the detection limit of 130 CFU/mL. As shown in Tables S2 and S3 (Supporting information), the cost of the supporting device was as low as $121, and the cost of reagent consumables for each test was $4.50. More importantly, this bacterial detection procedure, which integrated PMAxx labeling, nucleic acid extraction and RAA detection, did not rely on cumbersome instrument, professional technicians and complex operations. Thus, it might be a promising solution for on-site detection of foodborne bacteria under resource-limited conditions, after the magnetic beads are lyophilized and pre-stored in the chip to further simplify the procedure in our future work.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2022.03.083.

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