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Multichannel oscillatory-flow multiplex PCR microfluidics for high-throughput and fast detection of foodborne bacterial pathogens

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Abstract In the field of continuous-flow PCR, the amplification throughput in a single reaction solution is low and the single-plex PCR is often used. In this work, we reported a flow-based multiplex PCR microfluidic system capable of performing high-throughput and fast DNA amplification for detection of foodborne bacterial pathogens. As a demonstration, the mixture of DNA targets associated with three different foodborne pathogens was included in a single PCR solution. Then, the solution flowed through microchannels incorporated onto three temperature zones in an oscillatory manner. The effect factors of this oscillatory-flow multiplex PCR thermocycling have been demonstrated, including effects of polymerase concentration, cycling times, number of cycles, and DNA template concentration. The experimental results have shown that the oscillatory-flow multiplex PCR, with a volume of only 5 μl, could be completed in about 13 min after 35 cycles (25 cycles) at 100 μl/min (70 μl/min), which is about one-sixth of the time required on the conventional machine (70 min). By using the presently designed DNA sample model, the minimum target concentration that could be detected at 30 μl/min was 9.8×10^{-2} ng/μl (278-bp, *S. enterica*), 11.2×10^{-2} ng/μl (168-bp, *E. coli* O157: H7), and 2.88×10^{-2} ng/μl (106-bp, *L. monocytogenes*), which corresponds to approximately 3.72×10^{4} copies/μl, 3.58×10^{4} copies/μl, and 1.79×10^{4} copies/μl, respectively. This level of speed and sensitivity is comparable to that achievable in most other continuous-flow PCR systems. In addition, the four individual channels were used to achieve multi-target PCR analysis of three different DNA samples from different food sources in parallel, thereby achieving another level of multiplexing.

Keywords Oscillatory-flow · Multiplex PCR · Multi-channel · High-throughput · Continuous-flow · Detection of foodborne bacterial pathogens

1 Introduction

During the past decade, there has been an increased demand for rapid, high-throughput, and accurate detection of pathogenic bacteria, viruses, and other disease-causing agents. To meet this demand, the polymerase chain reaction (PCR) and its various variants, as a powerful molecular diagnosis tool, have been very widely developed and studied. Since it can offer advantages in terms of reduced assay time, low reagent consumption, high portability and integration of multiple processing modules, microfluidic PCR has attracted significant attentions from both academic and commercial areas (Auroux et al. 2004b; Zhang et al. 2006; Chen et al. 2007a; Zhang and Xing 2007). In this framework, DNA amplification and its upstream/downstream functional assays can be performed in a micro-scale reaction environment (such as a microchamber or microchannel).

Up to now, microfluidic PCR devices are mainly classified on the base of their operation in stationary or dynamic mode. Stationary PCR microfluidic devices

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(Northrup et al. 1993) are characterized by the use of single/multiple reaction chambers or microchannel regions. The PCR solution is introduced into microfabricated wells, and then the system (including the reaction solution) is subject to a repeated heating and cooling process according to a proper thermocycling profile. In this architecture, the large thermal mass usually determines high thermal inertial, thus leading to the relatively long cycling times. Instead, for dynamic devices, the PCR solution flows continuously through a microchannel incorporated into two/three isothermal zones. Only the reaction solution undergoes repeated heating and cooling, thus decreasing the relevant thermal inertial. As a result, the transition and residence times of the reaction solution in each temperature zone are related to the flow rate and thermal equilibrium time, enabling faster amplification process (Kopp et al. 1998).

Since its introduction (Kopp et al. 1998), dynamic continuous-flow PCR devices have been realized by different structure designs and different fluidic geometries (Frey et al. 2007; Ohashi et al. 2007; Chien et al. 2009; Hartung et al. 2009; Pjescic et al. 2010; Zhang and Xing 2010; Peham et al. 2011). Among them, the oscillatory-flow PCR devices have obtained increasing attention in recent years, due to their salient features such as cycle number/dwell time flexibility, ease of real-time detection implementation, large footprint reduction, and ability to process multiple samples in parallel. In 2001, Chiou et al. reported a close-cycle capillary PCR thermocycling machine, where the bidirectional pressure-driven flow and *in situ* optical position sensors have been integrated (Chiou et al. 2001, 2002). Using this device, 30 cycles of a 500-bp product were performed in 23 min with 78% amplification efficiency. Shortly afterwards, Day's group presented the first chip-based oscillatory-flow PCR microfluidic device (Auroux et al. 2003a,b, 2004a).

Since the introduction of these two works, the oscillatory-flow PCR devices have been well developed in the following aspects: (1) *The reactor's substrate material*. The microchannel of the oscillatory-flow PCR can be constructed in such substrates as silicon (Bu et al. 2003; Wang et al. 2005), glass (Sugumar et al. 2010), quartz (Baker et al. 2003), and various polymers (for example SU-8 (Auroux et al. 2003b, 2004a), poly(methylmethacrylate) (PMMA) (Hardt et al. 2004; Münchow et al. 2005; Cheng et al. 2005), cycloolefin copolymer (COC) (Hardt et al. 2004; Münchow et al. 2005), polydimethylsiloxane (PDMS) (Hu et al. 2006; Frey et al. 2007; Ugsornrat et al. 2008), polycarbonate (PC) (Ohashi et al. 2007), and dry film resist (DFR) (Kong et al. 2010)). To form a chip with sealed channels, these chips are required to bond with another cover plate. The materials for the cover chips include glass (Bu et al. 2003; Wang et al. 2005; Hu et al. 2006; Sugumar et al. 2010), quartz (Baker et al. 2003), PDMS (Frey et al. 2007), and PMMA (Auroux et al. 2003b, 2004a; Cheng et al. 2005). Each substrate material has different properties and therefore different advantages and disadvantages. It should be noted that the commercially available glass capillary has been used to produce the glass-PDMS hybrid chip for the oscillatory-flow PCR (Chen et al. 2007b; Polini et al. 2010; Sciancalepore et al. 2011). Compared with the all-PMDS chip, this hybrid configuration provides greatly reduced water evaporation, ease and low cost of fabrication, and conformal contact with the heater elements (Polini et al. 2010). (2) *The reactor's heater and sensor elements*. The heaters used for the oscillatory-flow PCR have changed from the metal/ceramic blocks with a certain heating cartridge (Chiou et al. 2001, 2002; Auroux et al. 2003a; Hardt et al. 2004; Münchow et al. 2005; Frey et al. 2007; Sugumar et al. 2010) to the Peltier heater (Chen et al. 2007b; Paik et al. 2007) and the film heaters integrated on the chips (for example indium tin oxide (ITO) (Cheng et al. 2005), platinum (Bu et al. 2003; Wang et al. 2005; Polini et al. 2010; Sciancalepore et al. 2011), chromium/aurum (Ugsornrat et al. 2008), and others (Baker et al. 2003; Auroux et al. 2003b, 2004a; Sista et al. 2008)). And, the temperature sensor elements include the commercially available thermocouples (Chiou et al. 2001, 2002; Auroux et al. 2003a; Chen et al. 2007b; Paik et al. 2007), the thermistors (Sista et al. 2008; Sugumar et al. 2010), and the on-chip film sensors (Bu et al. 2003; Wang et al. 2005; Polini et al. 2010; Sciancalepore et al. 2011). The use of integrated film heater and sensor can increase the degree of integration of the microfluidic device, although this may result in an increased cost of fabrication and may require special equipment and increased time for fabrication. It is worthy noting that Hu et al. have developed an electrokinetically controlled oscillatory-flow PCR in microchannel using Joule heating effect (Hu et al. 2006). In addition, Cheng et al. and Ohashi et al. have performed the oscillatory-flow PCR amplification on a linear temperature gradient device (Cheng et al. 2005; Ohashi et al. 2007). (3) *The control of fluid movement in microchannel*. The syringe pump is often used to drive the reaction solution to flow through a microchannel to realize the oscillatory-flow PCR (Auroux et al. 2003a,b, 2004a; Cheng et al. 2005; Wang et al. 2005; Chen et al. 2007b; Sugumar et al. 2010; Polini et al. 2010; Sciancalepore et al. 2011). The syringe pump is easy to use and simple in operation. However, it is usually bulky and expensive, which adversely affects the development of an integrated and portable oscillatory-flow PCR device. To overcome some of these drawbacks, some micropumping components have been developed for the oscillatory-flow PCR chips, such as the piezoelectric peristaltic micropump (Bu et al. 2003), the ferrofluidic magnetic micropump (Hardt et al. 2004; Münchow et al. 2005), the electrokinetic micropump

(Hu et al. 2006), the electrowetting micropump (Paik et al. 2007; Ugsornrat et al. 2008; Sista et al. 2008), the pneumatic micropump with an external actuator (Frey et al. 2007), and the droplet-based magnetic transportation (Ohashi et al. 2007). (4) *The detection sensitivity of the bidirectional flow reactor.* In the oscillatory-flow PCR devices, the ultrasensitive DNA detection has been obtained (Auroux et al. 2003b; Baker et al. 2003; Chen et al. 2007b; Sista et al. 2008). In 2003, Baker and co-workers reported an oscillatory-flow PCR chip device for high-throughput and single-molecule DNA amplification and analysis (Baker et al. 2003). Subsequently, Day's group presented a reliable and ultrasensitive oscillatory-flow DNA amplification microreactor for processing real-world genomic samples, with a detection limit of 24 human genome copies (Chen et al. 2007b). On the electrowetting-based digital microfluidic platform, the sample with one copy of *Candida* genome was successfully amplified and detected (Sista et al. 2008). These works demonstrate that the oscillatory-flow PCR has the great potential for performing rapid and sensitive DNA detection. (5) *The detection method associated with the bidirectional flow reactor.* All existing nucleic acid detection methods can be used for offline detection of oscillatory-flow PCR products. Possibly due to cost-effectiveness, the use of intercalators in combination with agarose gel electrophoresis is still the most popular method for detection of oscillatory-flow PCR products (Münchow et al. 2005; Wang et al. 2005; Cheng et al. 2005; Hu et al. 2006; Ohashi et al. 2007; Chen et al. 2007b; Sista et al. 2008; Sugumar et al. 2010; Polini et al. 2010; Sciancalepore et al. 2011). Offline product detection is usually time-consuming and labor intensive. Furthermore, it likely increases the risk of cross-contamination because of the manual sample loading. To circumvent these shortcomings, the real-time or endpoint fluorescence detection scheme has been used in the oscillatory-flow PCR devices (Baker et al. 2003; Hu et al. 2006; Frey et al. 2007; Chen et al. 2007b; Sista et al. 2008). As above-mentioned, the configuration of the oscillatory-flow microreactor is much suited to the application of the online detection strategy. Finally, it is worth emphasizing that the oscillatory-flow PCR functional unit has been integrated with the pre-amplification sample preparation and post-amplification product detection on a single chip (Baker et al. 2003; Hardt et al. 2004; Münchow et al. 2005; Sista et al. 2008), and thus the PCR-based, relatively total analytical systems can be constructed.

Although the oscillatory-flow PCR devices perform very well in the abovementioned aspects, they all only focus on the amplification of one DNA target in a single reaction solution (i.e. the oscillatory-flow single-plex PCR), and thus the detection throughput is low. For some cases where multiple DNA targets are required to be detected, such low throughput often results in a slower detection speed. As a result, for time-sensitive or throughput-sensitive diagnostics (e.g., detection of foodborne pathogens and related biowarfare agents), such diagnosis rate may lead to a failure in detection. In this work, we report an oscillatory-flow multiplex PCR for high-throughput DNA detection, where multiple DNA targets to be amplified are included in a single reaction solution. In addition, to make the best use of ease of access to the parallel multiple-sample analysis associated with the oscillatory-flow PCR, we also perform such oscillatory-flow multiplex PCR amplifications in multiple channels, and therefore the detection throughput is further increased. In our prototype, three DNA targets of different lengths are included in a single multiplex reaction solution for detection of three foodborne bacterial pathogens (*Listeria monocytogenes* (*L. monocytogenes*), *Escherichia coli* O157:H7 (*E. coli* O157:H7), and *Salmonella enterica* (*S. enterica*)). In addition, four reaction channels (including one negative-control channel) are used to simultaneously detect multiple bacterial pathogens in three different foods. The results show that the multichannel oscillatory-flow multiplex PCR presented here has great potential for applications in fast assays of a wide range of samples of diagnostic interest.

2 Design principle for the present high-throughput and fast DNA amplification method

In the oscillatory-flow PCR devices, there are two kinds of different channel designs: serpentine and straight. For the serpentine-channel oscillatory-flow PCR device (Cheng et al. 2005; Kong et al. 2010; Sugumar et al. 2010), enough heating and cooling of the reaction solution can be obtained at a certain flow rate. However, such design has some shortcomings: (1) the risk of bubble formation at the corner of the channel may be increased; (2) the use of the relatively long channel usually results in a sizable device footprint and an increased possibility of adsorption of biomacromolecules onto the channel inner surface; (3) the channel of a serpentine shape may limit the use of some pumping actuators in an oscillatory-flow PCR device; (4) finally, the use of the serpentine structure makes the channels difficult to arrange in parallel on a small footprint device. Based on these considerations, the straight reaction channels are used in this work. Figure 1(a) shows the schematic of design principle for the presented multichannel oscillatory-flow multiplex PCR. The n straight channels arranged in parallel to each other are incorporated onto three reaction zones those are maintained at constant temperatures for denaturation, annealing and extension. Within the multiplex PCR mixture in each channel, the m pairs of primers, together with the m kinds of DNA

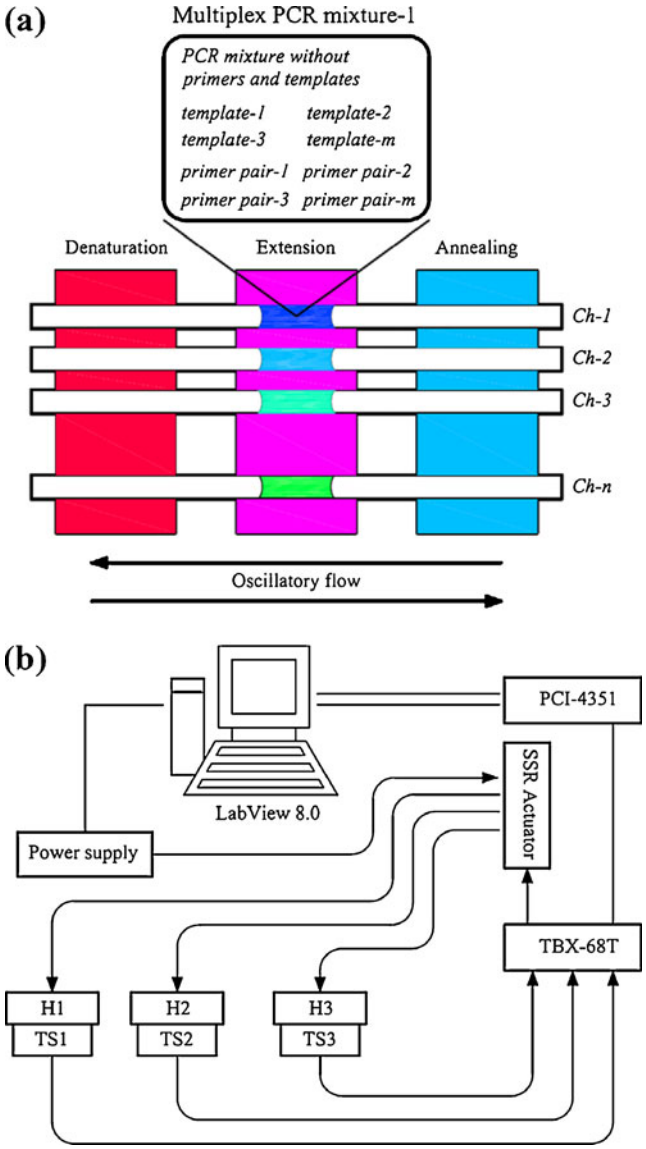

Fig. 1 (**a**) Schematic of design principle for multichannel oscillatory-flow multiplex PCR. (**b**) Schematic of the temperature measurement and control system for oscillatory-flow multiplex PCR. *H* heater, *TS* thermocouple sensor

templates, are included. When the number of primer pairs used in each reaction channel are same, the detection throughput of the present microfluidic device should be $m \times n$. Here, the DNA templates and primer pairs in each reaction mixture, and the number of the reaction channels can be flexibly selected to meet different application requirements. When the reaction mixtures in channels are driven to move back and forth between different temperature zones, the multichannel oscillatory-flow multiplex PCR can be realized. Here, it should be noted that the move of reaction mixture in channels can be simultaneously (Sun et al. 2008) or independently (Hua et al. 2010) controlled by a pumping mechanism. For the latter case, the cycle number of multiplex amplification, and the transition/dwell time of the reaction solution in each temperature zone may be different in each channel, which provides a wider range of applications.

3 Experimental section

3.1 Reagents, materials, and equipment

Chemicals for PCR mixtures, including Taq DNA polymerase (5 unit/μl), 10×Taq DNA polymerase buffer (100 mM Tris–HCl (pH 8.3), 15 mM $MgCl_2$, 500 mM KCl), and deoxynucleotide triphosphate (dNTPs) (2.5 mM each) were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Three pairs of primers were synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). And, the corresponding target genes/organisms, primer sequences, primer names, and product sizes are listed in Table 1, respectively. The target genes chosen in this study were the *hly* (hemolysin) for *L. monocytogenes*, the *rfbE* (O157-antigen) for *E. coli O157:H7*, and the *invA* (invasion protein A) for *S. enterica*, since they are described in the literature as being among the most specific and reliable genetic targets for the considered microorganisms. Three target organisms including *S. enterica* CMCC50040, *E. coli* O157:H7 GW1.0202, and *L. monocytogenes* CMCC54007 were obtained from Guangzhou Institute of Microbiology (Guangzhou, China).

Bovine serum albumin (BSA) (Fraction V, Purity ≥98%, Biotechnology Grade), which was utilized to dynamically passivate the inner surface to reduce the possible surface adsorption, was bought from Roche Diagnostics GmbH (Mannheim, Germany). Concentrated sulfuric acid, which could remove the possible PCR inhibitors from the channel before each run, was obtained from Tianjin Hongyan Chemical Reagent Factory (Tianjin, China). GoldView™ was purchased from SBS Genetech Co. Ltd. (Beijing, China). The DNA markers containing 500, 400, 300, 250, 200, 150, 100 and 50 bp fragments were from Dongsheng Biotech Co., Ltd. (Guangzhou, China). The TIANamp Bacteria Genomic DNA Extraction Kit was purchased from Tiangen biotech (Beijing) Co., Ltd. (Beijing, China). The polytetrafluoroethylene (PTFE) capillary (inner diameter (i.d.) 0.5 mm, outer diameter (o.d.) 0.9 mm) was purchased from Wuxi Xiangjian Tetrafluoroethylene Product Co. Ltd. (Wuxi, China). The 1,000-μl microliter syringe was bought from Shanghai Gaoge Industrial and Trading Co., Ltd. (Shanghai, China). The cartridge heater (100 W, 8 mm×40 mm) was produced by Guangzhou Haoyi Thermal Electronics Factory (Guangzhou, China).

The Mastercycler gradient PCR thermocycler (Eppendorf AG, Hamburg, Germany) is employed to perform the

Table 1 Sequences of forward and reverse primers for the desired target organisms to be detected by multiplex PCR

Target organism	Target gene	Primer	Sequence (5′-3′)	PCR product size (bp)
L. monocytogenes	*hlyA*	Lis-F Lis-R	GGGAAATCTGTCTCAGGTGATGT CGATGATTTGAACTTCATCTTTTGC	106
E. coli O157: H7	*rfbE*	Esc-F Esc-R	CAGTTTACCAACCGTCAT GAGCAACCGTTCCATTAC	168
S. enterica	*invA*	Sal-F Sal-R	AATTATCGCCACGTTCGG GCAA TCGCACCGTCAA AGGAACC	278

positive-control multiplex amplification reactions. The visualization of agarose gels is carried out by a gel imaging analysis system (Gel Doc XR, Bio-Rad, CA, USA). The BioPhotometer nucleic acid protein analyzer (Eppendorf AG, Hamburg, Germany) is used to test the concentration and purity of the extracted genomic DNA. The programmable precision syringe pump (CZ-74901-15, Cole Parmer, Vernon Hills, IL, USA), working in infusion and withdraw mode, is utilized for the manipulation of the reaction mixture. The homemade temperature measurement and control system (described below) is used to control the temperatures of three reaction zones in the oscillatory-flow multiplex PCR device. The temperature of the reaction liquid within the channels is also measured by a K-type thermocouple (0.005 inch diameter, Omega Engineering Inc., Stamford, CT, USA).

3.2 Bacterial culture, and DNA extraction and quantification for multiplex PCR

Three bacterial strains were used in this study to evaluate the present method and its applicability for detection of foodborne pathogens. All the bacterial strains were grown in Nutrient broth (0.3% beef extract, 1% soy peptone, and 0.5% NaCl) at 37°C overnight with rotary shaking at 200 rpm. The genomic DNA was extracted from 1 mL of overnight bacterial culture using the bacterial genomic DNA extraction kit following the manufacturer's instructions. The quantity and quality of the extracted DNA was determined by measuring A260 and the ratio of A260/A280 on the Eppendorf BioPhotometer. The concentrations of *S. enterica*, *E. coli* O157: H7, and *L. monocytogenes* DNA samples were 245, 280, and 72 ng/μl, respectively. The former two kinds of DNA samples have a high purity (A260/A280 is 1.8-1.9), but the *L. monocytogenes* DNA sample has a low purity (A260/A280 is about 1.4). To test whether the present method can be used to effectively detect the microbial pathogens in a food environment, the following sample model was also established. Three food samples including milk, banana, and hotdog were artificially contaminated with an overnight culture mixture: the 3-ml overnight culture mixture (1 ml each) was added in defined amounts of each food (10 ml milk, 10 g banana or 10 g hotdog). Then, 40 ml Nutrient broth was added to each artificially contaminated food sample and homogenized in a mortar, and 1 ml of the homogenate was subjected to DNA isolation.

3.3 Assembly of the oscillatory-flow PCR microfluidic device

The experiments were carried out using a computer-controlled, LabView-based temperature measurement and control system developed in our laboratory (Zhang and Xing 2010), which is one of the main components involved in the present oscillatory-flow multiplex PCR system. The schematic diagram of the systematic setup is shown in Fig. 1(b), and it mainly includes the following parts: three resistance cartridge heaters, three K-type thermocouple sensors (TS1, TS2, and TS3 in Fig. 1(b)), three grooved copper blocks hollowed out with one large circular hole, one small circular hole and four medial circular holes which were fabricated by Automation Engineering R&M Centre, Guangdong Academy of Sciences (Guangzhou, China), the PCI control module (PCI-4351, National Instruments Corp., Austin, TX, USA), the terminal block (TBX-68 T, National Instruments Corp., Austin, TX, USA), and the solid state relay (SSR) actuator module with a power supply unit. The cartridge heaters were inserted into the large circular holes of three copper blocks so as to form three heating zones (H1, H2, and H3 in Fig. 1(b)) for oscillatory-flow multiple PCR. The thermocouple sensors embedded within the small circular hole of the copper block were connected to the terminal block which is interfaced with the PCI control module. The temperature acquired with the thermocouple was used as the feedback signal for the fuzzy proportional/integral/derivative (PID) algorithm that was programmed in LabView™ (LabView 8.0, National Instruments Corp., Austin, TX, USA). To ensure good thermal contact of the cartridge heater/the thermocouple with the copper block, a small amount of thermal-conductive adhesives were applied between these contacting thermal elements. The photographs of the assembled oscillatory-flow PCR device

(including the syringe pump) and the LabView-based temperature control and measure system are shown in Fig. 2(a) and (b), respectively.

On the copper block, the groove channels were formed to perform multichannel oscillatory-flow multiplex PCR. Figure 3(a) shows the description of the actual parameters for the copper block. The copper block is 42 mm long, 24 mm wide, and 15.2 mm high, where ten parallel grooves of 1.2 mm width and 1.2 mm depth are formed, and cover an area of 24 mm×21 mm (i.e. the half of the upper surface area of the copper block). The diameters of the large, medial and small holes in the copper block are 8 mm× 42 mm, 3 mm×15.2 mm, and 1 mm×10 mm (diameter× length or depth), respectively. It should be noted that the small thermocouple hole within each copper block lies near the grooves just above the large hole (here, to make the figure clearer, the position and size of this thermocouple hole is not characterized). The groove channels on the copper block were covered with a thin glass cover whose edges are in contact with a raised piece of the copper block.

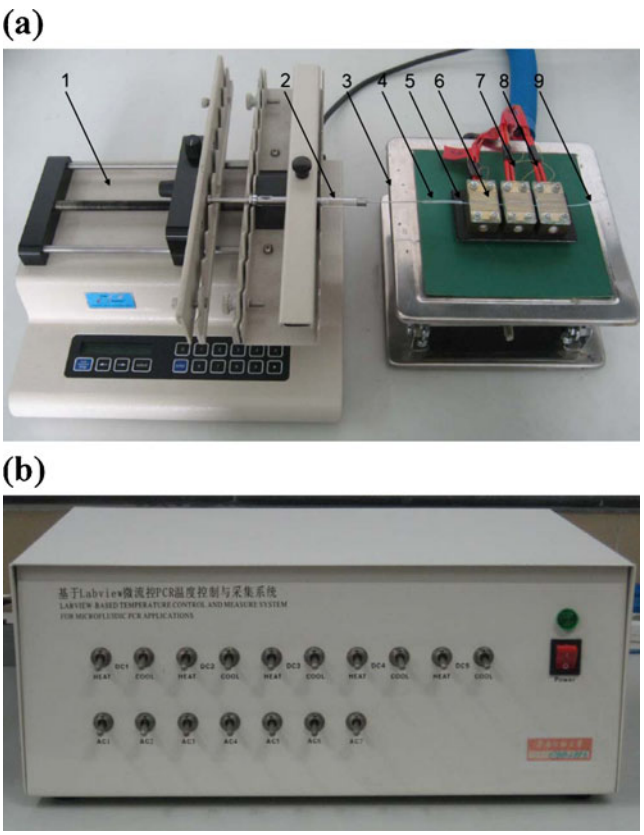

Fig. 2 Photographs of the assembled oscillatory-flow PCR microfluidic device (**a**) and the LabView-based temperature control and measure system (**b**). The microfluidic device shown in panel (**a**) include the precision syringe pump (1), glass syringe (2), lift table (3), silicon tube-based connector (4), support plate (5), copper block with a glass cover (6), thermocouple sensor (7), cartridge heater (8), and PTFE capillary tube (9)

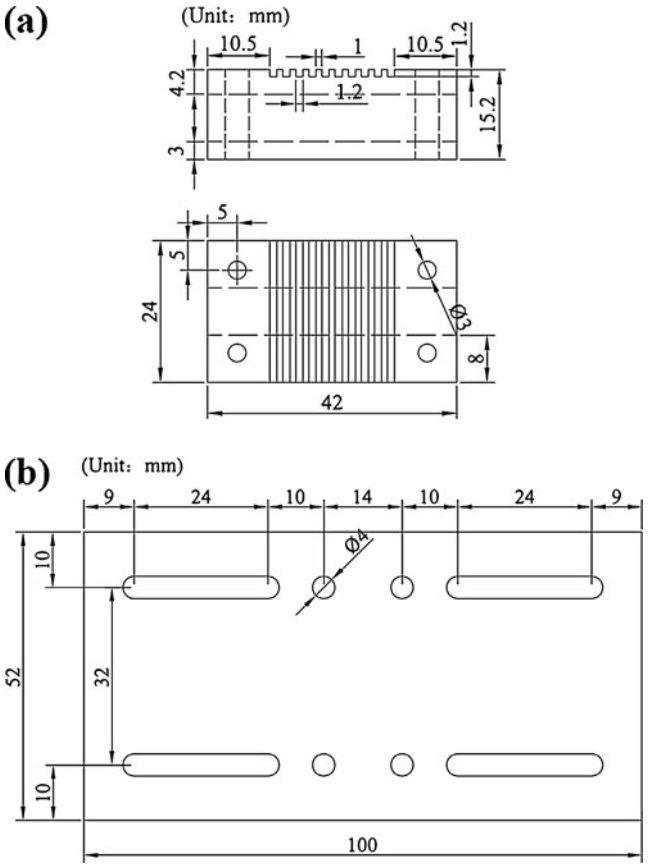

Fig. 3 Description of the actual parameters for the grooved copper block (**a**) and the support plate (**b**). In panel (**a**), the position and size of the thermocouple sensor's hole is not characterized in order to make the figure clearer

The glass cover provides heating from above and improves temperature uniformity inside the reaction channel. By using the four screws through the respective four circular holes, the heating copper block, along with a glass cover was fixed onto the support plate (Fig. 3(b)) to construct an oscillatory-flow multiplex PCR temperature zone. It is worthy noting that the extension temperature zone is firmly fastened on the middle of the support plate on which the four holes (4 mm in diameter) were used, but the denaturation/annealing-temperature copper blocks, along with the glass cover and four screws can flexibly move along the two straight tracks (24 mm (length)×4 mm (width)) on both sides of the support plate (Fig. 3(b)). When they move closest to the extension temperature zone, the air gap of about 0 mm between two adjacent temperature zones can be obtained. However, when they move farthest away from the extension temperature zone, the air gap of about 10 mm can be achieved. As a result, between two adjacent temperature zones, an air gap of greater than zero but less than 10 mm can be used to secure the desired thermal insulation. When an air gap of ~5 mm between two adjacent temperature zones is utilized, the

length of the capillary reactor is ~150 mm, including the lengths of the inlet/outlet part and the section connected to the syringe.

3.4 Operation of the microfluidic device for oscillatory-flow multiplex PCR

During experimental runs, the loading of the reaction solution was completed by using our devised sample loading scheme, as shown in Fig. 4. A capillary tube with suitable length was passed through a groove on the copper block that was covered by the glass piece, and then it was connected with a flat-tip syringe needle by a sleeve of silicon tube. In the presented sample loading method, the volumes of the reaction solution and the covering oil can be relatively precisely controlled by the programmable precision syringe pump. In Step 1 of Fig. 4, the reaction solution and mineral oil in the PCR tube was simply centrifuged (this helps the removal of air bubbles and delamination of the oil and solution), and then the capillary tube was inserted into the mineral oil layer manually. By running the syringe pump for a specified period of time, a certain volume of oil was pumped into the capillary tube. For Step 2, during the pump was stopped, the capillary tube was further inserted into the reaction solution layer manually. By the means similar to that in Step 1, the reaction solution with suitable volume was loaded into the capillary tube. In Step 3, the capillary tube was manually moved into the oil layer during the pump stopped. By running the syringe pump, then, a certain volume of oil was loaded into the capillary tube. After completion, the capillary tube was moved out of the PCR tube (Step 4), and the reaction solution flanked with the mineral oil plugs was driven into the oscillatory-flow reactor by the syringe pump. By using our devised sample loading scheme, no air bubble or plugs was formed between the reaction solution and the oil.

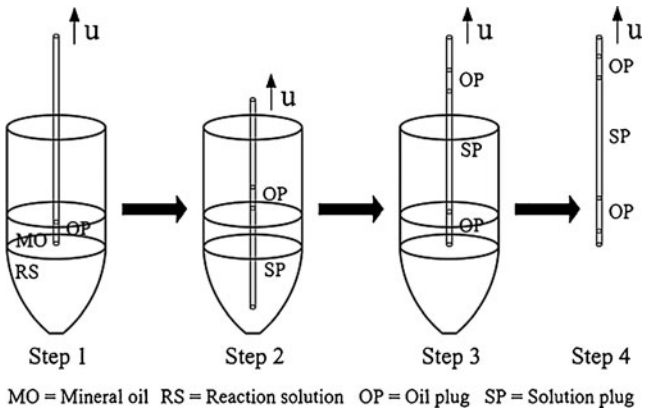

Fig. 4 Schematic of the reaction solution loading process in the oscillatory-flow multiplex PCR. Here, the *u* means the volume or linear flow rate designated on the syringe pump

Therefore, the reaction solution can be loaded from the inlet near the annealing zone with a temperature lower than 72°C or near the denaturation zone with a temperature higher than 90°C. These two kinds of processes do not result in the solution evaporation upon loading. After loading, to control the movement of the solution plug inside the channel, the pressure in the glass syringe with a volume of 1,000 μl was reduced to pull the solution forward through the channel. When the air inside the syringe was pressurized (by reversing the action of the pumping direction), the liquid plug was driven back toward the initial position inside the channel. Here, it should be noted that when multiple capillary tubes were used to perform multichannel oscillatory-flow multiplex PCR, the solutions in each tubes were driven by a single syringe pump, and the flow rates achieved in each tubes were similar (i.e., the reaction solutions can not be independently run and controlled for individual tubes). After oscillatory-flow multiplex PCR, the reaction solution together with the oil plugs was driven out of the capillary tube, and flowed into the PCR tube. By a simple centrifugation, the reaction solution was separation from the oil.

3.5 Multiplex PCR using an oscillatory-flow microreactor

The following experimental conditions were used to evaluate the performance of the oscillatory-flow multiplex PCR platform. The multiplex PCR mixture contained 2× Taq DNA polymerase buffer, 0.3 mM each of the dNTPs, 0.4 μM each of the primers, and 0.5 μg/μl BSA. Within the reaction cocktail, unless otherwise stated, the concentrations of *S. enterica*, *E. coli* O157: H7, and *L. monocytogenes* DNA samples were 9.8, 11.2, and 2.88 ng/μl, respectively; the Taq DNA polymerase was 0.2 unit/μl in concentration. After being introduced into the capillary channel, an about 5 μl multiplex PCR solution, together with two oil plugs (1–2 μl), was driven back and forth by the syringe pump. To easily observe the movement of the multiplex PCR solution, the PCR-biocompatible bromophenol blue was also added into the reaction solution. Here, it needs to note that the reaction solution continuously flowed through each zone, and thus the multiplex amplification occurred during a continuous flow process. The main reason for doing it is that the amplification length of each target gene fragment in the present microfluidic system is relatively small, and therefore the time it takes for the solution to flow through the extension zone may be enough to induce the multiplex extension reaction. In addition, it is possible that the primer extension reaction occurs when the reaction solution is inside of the annealing heat zone and between the annealing and extension heat zones (annealing takes places as the reaction solution travels between heat zones) (Chiou et al. 2001, 2002; Chen et al. 2007b). Unless

demonstrated otherwise, the thermal cycles of 35 were used for the oscillatory-flow multiplex PCR, and the flow rate of the reaction liquid was 30 μl/min. To verify the performance of the oscillatory-flow multiplex PCR, the positive-control multiplex PCR was carried out on the conventional PCR instrument. The thermocycling protocol consisted of an initial incubation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s, and a final incubation at 72°C for 2 min, with a total run time of 70 min including temperature ramp times.

3.6 Analysis of amplification products

After the reactions were completed, the products were analyzed by the agarose gel electrophoresis and GoldView™ dye prestaining method. The gel was prepared with 2% (w/v) agarose in 20 mL of 1× TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.5) containing 1 μL of GoldView™ dye. The gel was run for approximately 30 min at 100 V, and then the fragments were visualized under UV light on the gel imaging analysis system. And, the relative amounts of multiplex PCR products were analyzed by the image analysis software (Quantity One, Bio-Rad, CA, USA). The DNA markers were used as the evaluation standards for the amplified DNA fragments.

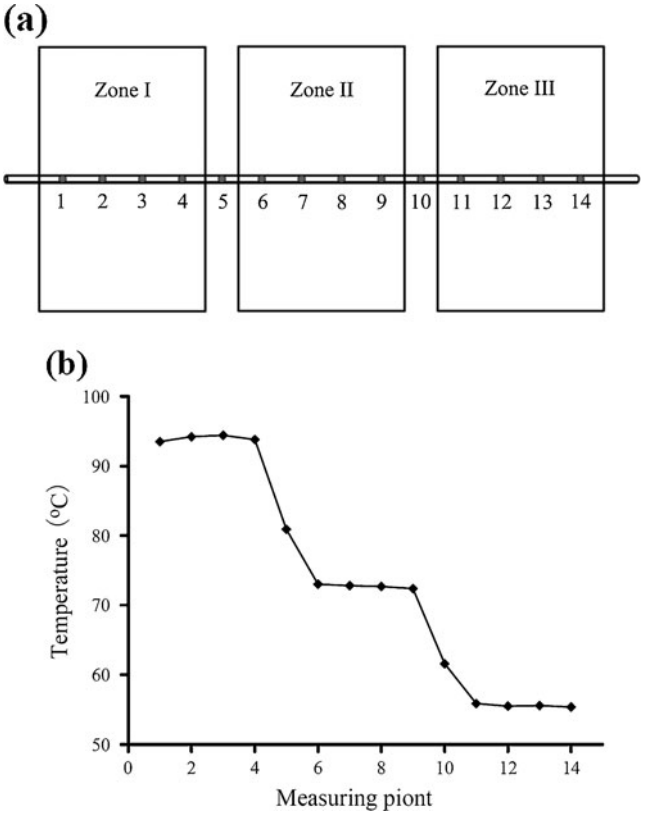

Fig. 5 (**a**) Schematic drawing of the temperature measuring points along the reaction microchannel. (**b**) Temperature distribution in the reaction microchannel. Data at each point was measured five times

4 Results and discussion

4.1 Evaluation of the oscillatory-flow thermocycling

Since PCR is very much temperature-sensitive, before performing any multiplex amplification experiments it is necessary to first evaluate the performance of the temperature control in the oscillatory-flow multiplex PCR device. In the present work, multiplex PCR was carried out in a capillary channel embedded into the grooves on the heating copper block, and thus the temperature distribution of the capillary channel was investigated. To gain a relatively accurate temperature measurement of the fluid within the channel, the miniature thermocouple was inserted into the capillary channel filled with the 1×Taq DNA polymerase buffer solution. During these experiments, no bubble formation was observed at the three temperature regimes involved in the oscillatory-flow multiplex PCR. Figure 5(a) shows the points along the capillary microchannel where temperature was measured. Points 1–4 were located in the denaturation zone (Zone I), points 6–9 in the extension zone (Zone II), points 11–14 in the annealing zone (Zone III), points 5 and 10 in between two adjacent zones. The three zones were maintained at 94, 72, and 56°C, respectively. Figure 5(b) shows the temperatures measured along the microchannel. It is clear that three different temperature zones were formed. In each zone, the respective temperature was almost constant, changing sharply between the two adjacent zones. This is likely because of the proper thermal isolation by air gaps and the low thermal conductivity of PTFE. As the cycling temperatures were precisely controlled, desirable temperature kinetics for oscillatory-flow PCR could be obtained. It should be noted that other DNA templates with various temperature requirements can also be amplified by simply changing the temperature setting. In addition, it needs to point out that the thermocouple-based temperature measurement proposed here is an indirect measurement of the solution temperature by taking the metal block temperature. To acquire the direct temperature information of the samples in a 0.5-mm diameter reaction tube without causing massive disruption on flow inside the tube, the numerical simulation approaches can be used, such as finite element analysis (FEA) (Bu et al. 2003; Wang et al. 2005; Hu et al. 2006; Ugsornrat et al. 2008). Also, the noninvasive infrared (IR) thermography-based technique can be utilized to non-invasively measure the temperatures for oscillatory-flow PCR (Cheng et al. 2005; Kong et al. 2010).

For the small-volume oscillatory-flow PCR systems, solution evaporation is often problematic. To circumvent such evaporation, some measures have been taken. Within Cheng et al.'s oscillatory-flow PCR chip, a single opening was used for both sample loading and syringe pump port (Cheng et al. 2005). When the solution plug was pumped to high-temperature zones, the internal pressure increased by six times and therefore the evaporation was significantly decreased. However, their approach may add more requirements to the chip package. In addition, this will increase the possibility of cross-contamination occurring. In the oscillatory-flow PCR systems reported so far, the widely used way for reducing solution evaporation is the use of the mineral oil cover as a vapor barrier. The mineral oil is a suitable liquid cover since it has a boiling point far above 100°C and a density slightly below 1.0 g/cm^3. However, special care is often taken to avoid the presence of air bubbles (or air plugs) between the oil cap and the reaction solution during sample loading. In this study, we have devised a sample loading scheme to reduce the possibility of the formation of air plugs between them (Fig. 4). By this approach, about 5 μl of the reaction solution that was well capped by two oil plugs (1–2 μl) could be conveniently introduced into the reaction channel. By using the oil caps to seal the reaction solution, we found no appreciate solution reduction after 35 cycles. In addition, by introducing a highly viscous mineral oil at two ends of the reaction solution, the formation of bubbles at elevated temperatures could be escaped. This is likely because the used oil in return helps increase the pressure of the reaction solution in the channel, which prevents the formation of bubbles.

4.2 Effects of polymerase concentration, cycling times, number of cycles, and DNA template concentration on oscillatory-flow multiplex PCR amplification

After a preliminary setting of suitable primer concentrations, master mixture composition and temperature conditions, the amplification capability of oscillatory-flow multiplex PCR was evaluated. Before this evaluation, however, it is essential to introduce the presently designed DNA sample model: (1) the DNA samples were extracted from different types of foodborne bacterial pathogens. The *S. enterica* and *E. coli* O157: H7 are the Gram-negative bacteria which are the two most important pathogens causing human infectious diseases, while *L. monocytogenes* is a kind of Gram-positive bacterium that is known as a virulent, hard-to-kill pathogen that can survive at refrigeration temperatures, and that can become rooted in food processing environments and persist for a long time, even years; (2) within the DNA samples to be used, the concentration of each DNA sample is different. Moreover, the purity of the respective DNA sample is also different (for *S. enterica* and *E. coli* O157: H7 DNA samples, A260/A280 is 1.8-1.9; for *L. monocytogenes* DNA sample, A260/A280 is 1.4 or so). The purpose of this design is to make the DNA samples in the multiplex PCR close to those extracted from the real-world foods contaminated with bacterial pathogens. In those real-world contaminated foods, the types of bacterial pathogens and their respective concentrations may be different. Therefore, the concentration of each bacterial DNA may be different when a similar genomic DNA extraction approach was used. As a result, the extracted DNA sample's purity is usually relatively low.

The present multiplex PCR was performed in a standard 0.5 mm i.d capillary with a wall thickness of 0.2 mm, and the surface-to-volume ratio (SVR) of this channel reactor is several times larger than that of the conventional PCR tube, which will cause the more bio-molecules, especially Taq DNA polymerase and DNA template, to be adsorbed onto the inner surface. In a multiplex PCR, in addition, more amount of reagents (for example Taq DNA polymerase and DNA templates), reaction time (for example extension time), and even number of cycles are usually required because more than one target fragments are simultaneously amplified (Henegariu et al. 1997). Therefore, the effects of these factors on the oscillatory-flow multiplex PCR were evaluated here. Figure 6(a) shows the influence of Taq DNA polymerase concentration in the oscillatory-flow multiplex PCR. It is clear that the multiplex amplification efficiency with high concentration of Taq DNA polymerase (for example 0.2–0.5 unit/μl, lanes 5–8 in Fig. 6(a)) appeared higher than that with a lower concentration of Taq DNA polymerase using the same oscillatory-flow multiplex PCR protocol. The similar experiments were also carried out on the conventional PCR machine (see electronic supplementary material (ESM), Fig. S1). In these experiments, when a wide range of Taq DNA polymerase concentrations changing from 0.025 to 0.5 unit/μl was utilized, the resulting multiplex PCR products could all be observed, although the band intensities of the corresponding multiplex PCR product decreased gradually. The comparison of the corresponding band intensities in Fig. 6(a) and Fig. S1 reveals that within the presented oscillatory-flow multiplex PCR, there still is greater possibility of adsorption of Taq DNA polymerase molecules onto the tubing inner surface, although the optimized 0.5 μg/μl BSA has been added into the reaction solution to dynamically coat the reaction channel (ESM, Fig. S2). In addition, this comparison also shows that a relatively high Taq DNA polymerase concentration was needed in the present oscillatory-flow multiplex PCR microfluidic system, likely due to the absorption of the enzyme to the capillary walls. To resolve these problems, the present BSA

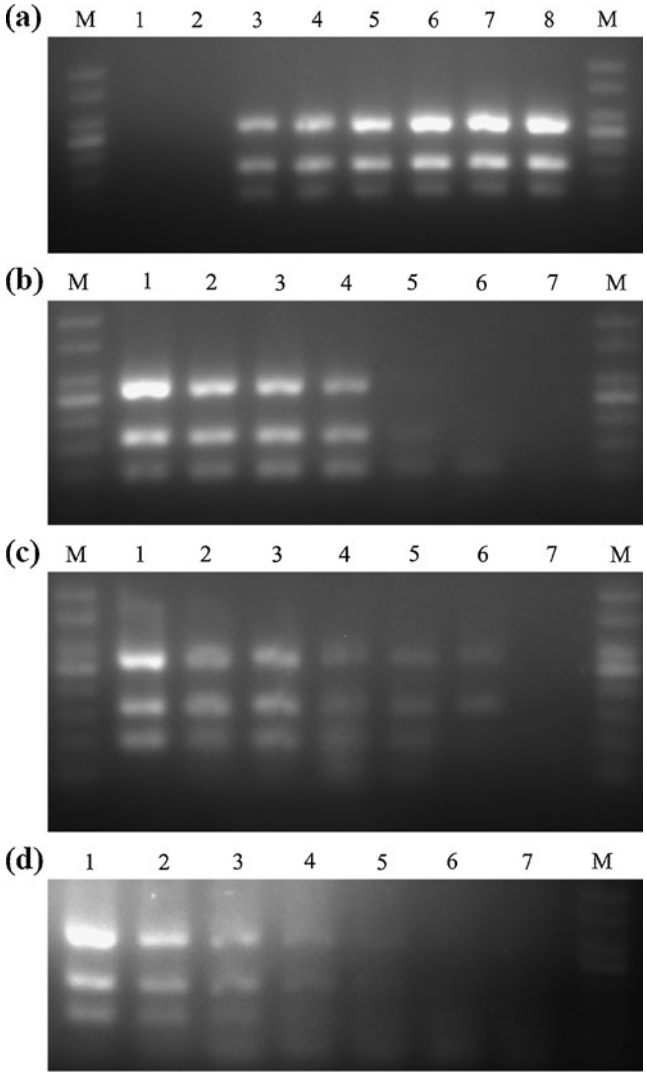

Fig. 6 DNA amplification using the oscillatory-flow multiplex PCR. (**a**) Influence of Taq DNA polymerase concentration in the oscillatory-flow multiplex PCR. Lane **M**: the DNA marker; Lanes **1**–**8**: the Taq DNA polymerase concentrations used in the oscillatory-flow multiplex PCR were 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 unit/μl, respectively. (**b**) Influence of the flow rate of the multiplex PCR solution in the channel on the oscillatory-flow multiplex PCR. Lane **M**: the DNA marker; Lane **1**: the positive-control multiplex PCR product; Lanes **2**–**6**: the oscillatory-flow multiplex PCR results were obtained at the flow rates of 50, 70, 100, 150, 200 μl/min, respectively;Lane **7**: the negative-control multiplex PCR. (**c**) Influence of the cycle number on the oscillatory-flow multiplex PCR yield. Lane **M**: the DNA marker; Lane **1**: the positive-control multiplex PCR product; Lanes **2**–**6**: the oscillatory-flow multiplex PCR was performed with the cycle number of 40, 35, 30, 25, and 20, respectively;Lane **7**: the negative-control multiplex PCR. During runs, the oscillatory-flow reaction solution flowed at the flow rate of 70 μl/min. (**d**) Influence of the input DNA template concentrations on the oscillatory-flow multiplex PCR. Lane **M**: the DNA marker; Lane **1**: the positive-control multiplex PCR product; Lane **2**: the concentrations of *S. enterica*, *E. coli* O157: H7, and *L. monocytogenes* DNA templates were 9.8, 11.2, and 2.88 ng/μl, respectively; Lanes **3**–**6**: the concentrations of the used DNA template samples were the 10, 100, 1,000, 10,000-fold dilutions of the initial DNA template concentration (as stated in Lane **2**); Lanes **7**: the negative-control multiplex PCR

passivation may be required to couple with other surface treatments (Zhang et al. 2006; Zhang and Xing 2007). In Fig. 6(b), the effect of different flow rates of the reaction solution on the oscillatory-flow multiplex PCR was tested. When the flow rate was 100 μl/min, three target DNA fragments were successfully amplified in about 13 min after 35 cycles, and this time is about one-sixth of the time required on the conventional PCR machine (70 min, including the times of each PCR step and temperature ramping times). Therefore, the present multiplex PCR system is much faster that the conventional PCR machine. This is mainly attributed to the rapid temperature transition and the reduced channel length associated with the present oscillatory-flow PCR reactor. Compared with the serpentine or spiral channel continuous-flow PCR systems, the oscillatory-flow PCR system can provide a flexible cycle number by only modifying the pumping program, without need to fabricate a new device. In addition, when time-sensitive diagnostics (e.g., detection of infectious diseases and related biowarfare agents) is needed, the PCR may be performed with a moderate cycle number so as to shorten the total detection time. Therefore, the effect of cycle number on the oscillatory-flow multiplex PCR was also studied. Figure 6(c) displays this effect, where the relative high flow rate of 70 μl/min was used and tested. As seen from Fig. 6(c), the most efficient cycle number seemed to be around 35. Too many thermal cycles may result in the reduced amplification efficiency (lane 2 in Fig. 6(c)). This can be attributed to the extended stay of the Taq enzyme at high temperatures (such as 94°C) in the denaturation step, which may inactivate the enzyme resulting in lower amplification yields (Schneegaβ et al. 2001). In addition, excessive number of cycles will increase the non-specific amplification products which may be hard to be distinguished from the agarose gel electrophoresis, leading to the decreased PCR yield of the target DNA fragments. With the decrease of cycle number from 35 to 20, the oscillatory-flow multiplex PCR yield was gradually reduced. Of course, lower yields with lower cycle numbers are a common finding in the continuous-flow PCR (Obeid et al. 2003) and even conventional PCR systems. When the cycle number used was 25, the multiplex amplification product was small in yield but could still be distinguished by the gel electrophoresis pattern (lane 5 in Fig. 6(c)), where amplification was completed in about 13 min that was 3 min shorter than the time required at 35 thermal cycles in the case of 70 μl/min. It should be noted that under other similar conditions, when the flow rate of 50 μl/min was used, 20 cycles could be indicated as the minimum of cycles to realize a successful oscillatory-flow multiplex PCR, where amplification was accomplished in about 14 min which was 7 min shorter than that required in the case of 35 thermal cycles

(ESM, Fig. S3). Therefore, the minimum number of cycles to obtain a desired oscillatory-flow multiplex PCR depends to a certain extent on the flow rate of the amplification solution in a microchannel. For some applications, it may be important to obtain a detectable amount of multiplex PCR product relatively quickly from a low-abundant sample. Therefore, a study was carried out to establish the minimum concentration of template that could be detected when amplified through 35 thermal cycles at the flow rate of 30 μl/min, as shown in Fig. 6(d). It needs to be noted here that the use of more thermal cycles and lower flow rate can increase multiplex PCR yield from a low-abundant target sample to facilitate a sensitive multiple PCR detection. By the presently built DNA sample model, the minimum template concentration that could be simultaneously detected within a single reaction microchannel at the flow rate of 30 μl/min was 9.8×10^{-2} ng/μl (*S. enterica*), 11.2×10^{-2} ng/μl (*E. coli* O157: H7), and 2.88×10^{-2} ng/μl (*L. monocytogenes*), which corresponds to approximately 3.72×10^{4} copies/μl (*S. enterica*), 3.58×10^{4} copies/μl (*E. coli* O157: H7), and 1.79×10^{4} copies/μl (*L. monocytogenes*). This sensitivity of detection is acceptable in most of applications, but it is about two orders of magnitude lower than that obtained by testing the microfluidic system with the relatively pure DNA samples which were extracted by using the bacterial DNA extraction kit with a lysozyme digestion (for each DNA sample, A260/A280 was 1.8–1.9) (ESM, Fig. S4). Therefore, the relatively low detection sensitivity obtained in the present microfluidic system is most likely attributed to the impurity of the used *L. monocytogenes* DNA sample. Finally, it should be noted that the experiments in Fig. 6 were all repeated three times with excellent reproducibility, indicating the robustness of the oscillatory-flow multiplex PCR system.

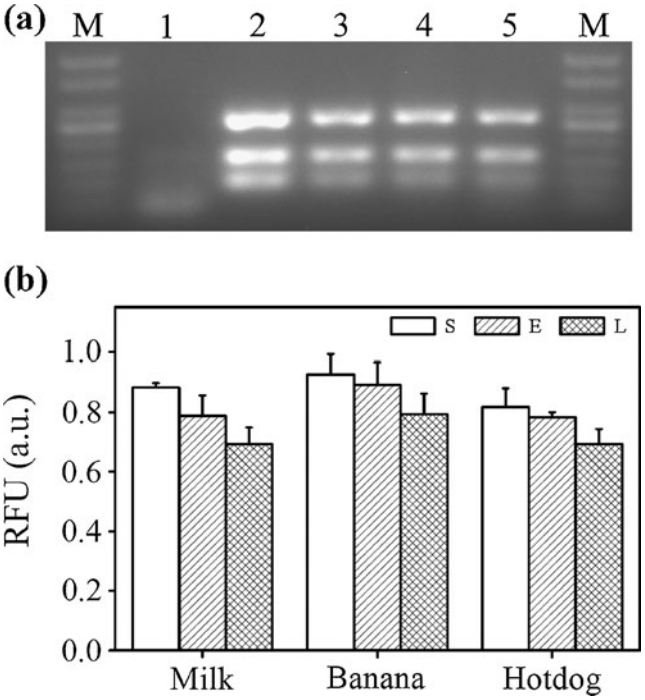

Fig. 7 Detection of three foodborne pathogens in milk, banana and hotdog using the multichannel oscillatory-flow multiplex PCR. (**a**) Gel electrophoresis analysis of multiplex PCR products of DNA targets from three foodborne bacterial pathogens in different foods. Lane **1**: the negative-control multiplex PCR; Lane **2**: the positive-control multiplex PCR; Lanes **3**–**5**: the oscillatory-flow multiplex PCR of DNA targets from three bacterial pathogens in milk (lane 3), banana (lane 4) and hotdog (lane 5). (**b**) Comparison of band intensities of the oscillatory-flow multiplex PCR products (lanes 3–5 in panel (**a**)) with those of conventional multiplex PCR products (lane 2 in panel (**a**)), which were analyzed by the image analysis software (Quantity One). The fluorescence intensities of the former's bands were normalized to those of the product's bands from the conventional PCR machine (100%, lane 2). Here, the experiments were independently done three times. S-*Salmonella enterica*, E-*Escherichia coli* O157:H7, L- *Listeria monocytogenes*

4.3 Multichannel oscillatory-flow multiplex PCR for simultaneous detection of multiple bacterial pathogens in three different foods

To test whether the present method can be used to detect the microbial pathogens in food, three different foods (milk, banana, and hotdog) were artificially contaminated with a bacterial culture mixture (~10^{8} cfu/ml each bacterium), and then the extracted DNA mixture was subjected to multiplex PCR amplification. During runs, three reaction channels (*Ch-3*, *Ch-6*, and *Ch-9*, as shown in Fig. 1(a)) were used to amplify and detect the bacterial DNA targets extracted in milk, banana, and hotdog, respectively. And, the *ch-1* reactor was always used to perform the negative-control oscillatory-flow multiplex PCR without any DNA templates. The crosswise experiments were independently performed three times in these three channels (i.e. the *Ch-3-Ch-6-Ch-9*, *Ch-6-Ch-9-Ch-3*, and *Ch-9-Ch-3-Ch-6* reactors were used for the bacterial detection in milk, banana, and hotdog, respectively). Figure 7(a) shows a typical gel electrophoresis analysis of multiplex PCR products of three bacterial DNA samples in different foods (lanes 3–5 in Fig. 7(a)). Lane 2 in Fig. 7(a) denotes the positive-control multiplex PCR product amplified from the DNA templates extracted from the bacterial cultures, as similar to the cases in Fig. 6. Figure 7(b) displays the analytical results of three independent multiplex amplification experiments. Here, the image analysis software (Quantity One) was used to quantify and compare the amounts of multiplex PCR products of the oscillatory-flow microfluidic system relative to those of the conventional PCR machine. Seen from Fig. 7(b), the present multichannel oscillatory-flow multiplex PCR system could simultaneously detect multiple bacterial pathogens in three different foods, and moreover its detection performance was comparable to that of the

conventional multiplex PCR. For example, the average relative fluorescence intensities obtained for simultaneous detection of three different bacterial pathogens in three different foods were about 0.82–0.93, 0.78–0.89, 0.69–0.79 for *S. enterica*, *E. coli* O157: H7, and *L. monocytogenes*, respectively. In addition, the multiplex amplification in the present microfluidic system was well reproducible, because the standard deviation values labeled in Fig. 7(b) were relatively small as compared with the corresponding average values. As expected, however, in each case the 106-bp product for detection of *L. monocytogenes* pathogens was relatively low in yield. This is likely because the DNA extraction kit used presently could more effectively extract DNA from the Gram-negative bacteria than from the Gram-positive bacteria. In general, the cell wall of the Gram-positive bacterium is difficult to break down. To effectively lyse the Gram-positive bacteria to obtain more pure DNA sample, the lysozyme is usually used. As seen from Fig. S4, when the relative pure DNA sample extracted by the current DNA extraction kit with a preliminary lysozyme digestion, was used as the oscillatory-flow PCR template, the amplification yield of Gram-positive *L. monocytogenes* gene fragment could be relatively increased under other similar conditions.

5 Conclusions

In conclusion, we have developed an oscillatory-flow multiplex PCR system to achieve the high-throughput and fast nucleic acid amplification. It has been shown that the developed system is capable of performing multi-target PCR detection, exhibiting remarkable detection ability by amplifying the multiplex mixture of bacterial DNA targets with a relatively low purity. Under stable, controlled conditions, the 35-cycle (25-cycle) oscillatory-flow multiplex PCR could be accomplished in about 13 min when the flow rate of 100 μl/min (70 μl/min) was used. Under similar conditions, 20 cycles could be completed in about 14 min at 50 μl/min. Therefore, the oscillatory-flow multiplex PCR time is several times shorter than that required on the conventional PCR instrument. Using the presented DNA sample model, the minimum target concentration that could be detected at 30 μl/min was 3.72×10^4, 3.58×10^4, and 1.79×10^4 copies/μl for *S. enterica*, *E. coli* O157: H7, and *L. monocytogenes*, respectively. The speed and sensitivity of the assay is comparable to that obtained using the single-plex PCR in other continuous-flow systems. In addition, as proof of principle, the four-channel oscillatory-flow multiplex PCR was performed to analyze multiple DNA targets from different foods in parallel, thus adding another level of multiplexing.

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