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Rapid detection of genetically modified organisms on a continuous-flow polymerase chain reaction microfluidics

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ABSTRACT

The ability to perform DNA amplification on a microfluidic device is very appealing. In this study, a compact continuous-flow polymerase chain reaction (PCR) microfluidics was developed for rapid analysis of genetically modified organisms (GMOs) in genetically modified soybeans. The device consists of three pieces of copper and a transparent polytetrafluoroethylene capillary tube embedded in the spiral channel fabricated on the copper. On this device, the P35S and Tnos sequences were successfully amplified within 9 min, and the limit of detection of the DNA sample was estimated to be 0.005 ng μl^{-1}. Furthermore, a duplex continuous-flow PCR was also reported for the detection of the P35S and Tnos sequences in GMOs simultaneously. This method was coupled with the intercalating dye SYBR Green I and the melting curve analysis of the amplified products. Using this method, temperature differences were identified by the specific melting temperature values of two sequences, and the limit of detection of the DNA sample was assessed to be 0.01 ng μl^{-1}. Therefore, our results demonstrated that the continuous-flow PCR assay could discriminate the GMOs in a cost-saving and less time-consuming way.

Since the beginning of recombinant DNA technology, genetically modified organisms (GMOs)[1] have brought many advantages such as considerable improvement in the yield and quality of crops and enhancement of the nutritional quality of plants [1]. With the widespread use of GMOs in food production, labeling regulations have been established in some countries to protect the rights of consumers, producers, and retailers [2]. For example, the European Union has been regulating the labeling of genetically modified (GM) foods since 1997 (regulation 258/97/EC).

To verify compliance with labeling requirements, several systems for the detection of GMOs have already been developed and described [3]. Up to now, the detection molecules of GMOs have included DNAs, RNAs, and proteins [4–6]. DNA is a relatively stable molecule, allowing its extraction from all kinds of tissues due to uniqueness of DNA in every type of cell and its analysis from processed and heat-treated food products. Therefore, polymerase chain reaction (PCR) [7] based on detection of DNA is the most widespread method [8,9]. For example, the matrix approach proposed by INRA (French National Institute for Agriculture Research) in 1999 for the GMOchips program was a combination of PCR and hybridization to detect authorized and unauthorized GMOs [10]. Currently, a large number of GMOs share the same promoter of the subunit 35S of ribosomal RNA of cauliflower mosaic virus (P35S) and the nopaline synthetase terminator (Tnos) from *Agrobacterium tumefaciens* [11]. Thus, in practice, they are widely amplified to detect whether the tissues contain GM components.

Today most PCR amplifications are carried out on a conventional PCR machine using a heating/cooling block of large heat capacity that often has a number of technical frailties and ultimately restricts the speed and efficiency of the amplification process [12–15]. For rapid PCR, the low heat capacity of the entire PCR system is important, and performing the rapid PCR is difficult in a conventional PCR instrument using a heating/cooling block of large capacity. Therefore, some research groups have made an attempt to develop microfluidics-based PCR biomicrofluidic devices [16,17]. Currently, there are two formats of microfluidic PCR devices [18–20]: microchamber stationary PCR and continuous-flow PCR. The former is the miniaturization of conventional PCR in nature where the PCR mixture is stationary in the chamber and the temperature is cycled repeatedly [21–23]. However, the chamber stationary PCR microfluidics lacks the flexibility to change the

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[1] *Abbreviations used:* GMO, genetically modified organism; GM, genetically modified; PCR, polymerase chain reaction; P35S, 35S promoter; Tnos, the nopaline synthetase terminator; μ-TAS, micro total analytical system; MCA, melting curve analysis; PID, proportional/integral/derivative; PTFE, polytetrafluoroethylene; dNTP, deoxynucleotide triphosphate; ddH_2O, doubly deionized H_2O; BSA, bovine serum albumin; BPB, bromophenol blue; EDTA, ethylenediaminetetraacetic acid; CV, coefficient of variation; SD, standard deviation; SVR, surface-to-volume ratio; LOD, limit of detection; ELISA, enzyme-linked immunosorbent assay; RT–PCR, real-time PCR.

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reaction rate, resulting in more cycling and heating time. Moreover, to reduce the reaction time and power consumption, the system thermal mass must be optimized considerably [24]. Compared with microchamber stationary PCR, the continuous-flow PCR has a few advantages [25]. The heating and cooling rates for PCR amplification are confined not by the system thermal mass but rather by the flow velocities of PCR mixture in a microchannel, the PCR sample solution does not suffer from large evaporation at high temperatures, it is easier to integrate other analytical elements to develop the micro total analytical system (μ-TAS) [26,27], and this format can realize high-throughput PCR amplification by continuously providing various biological sample plugs that could save much time and labor. Currently, the structural styles of the continuous-flow PCR microfluidics can be divided into three main categories: (i) the serpentine channel continuous-flow PCR [25,28–34], a continuous-flow format that is based on the work of Nakano and coworkers [17] and Kopp and coworkers [28]; (ii) the spiral channel continuous-flow PCR [35–41], which consists of a "circular" arrangement of the three zones to generate the sequence of denaturation, annealing, and elongation; and (iii) the straight channel oscillatory-flow PCR [42–45], a type of flow-through PCR microfluidics that consists of a capillary tube, heater zones, an optical window, and so on.

In complying with ISO/DIS 24276, successive simplex DNA amplification and duplex PCR were used in the study for GMO detection to save considerable time and effort. For the purpose of identifying the 195- and 180-bp sequences simultaneously, melting curve analysis (MCA) was exploited in duplex PCR, which was based on SYBR Green I, an intercalating dye that is widely used [46]. With a high affinity for double-stranded DNA and enhanced fluorescence on DNA binding [47], SYBR Green I offers a good alternative as continuous monitoring of the fluorescence of amplicons along with gradient changes in temperature. That can be used to determine the melting curves of these sequences. Although fluorescence in the SYBR Green I reaction is not sequence specific, it is possible to identify the amplified products by their melting temperature (*Tm*). Because the melting curve of a product is dependent on GC content, length, and sequence, PCR products can be distinguished by MCA [48].

Materials and methods

Apparatus

The continuous-flow PCR microfluidics device, depicted schematically in Fig. 1, was manufactured by Automation Engineering R&M Centre (AERMC, Guangdong Academy of Sciences, Guangzhou, China). It consists of a 4-cm diameter and 10-cm length copper cylinder machined into three pieces corresponding to the denaturation, annealing, and extension regions. The extension region is twice the size of the other two zones. The three temperature zones are separated from each other by thermally insulating sheets that have a thickness of 3 mm. Each zone includes one larger central hole (8 mm diameter) for the resistance cartridge heater (8 mm diameter, 100 mm length, 300 W, Guangzhou Haoyi Thermal Electronics Factory, Guangzhou, China) and two small holes (1 mm diameter and 10 mm depth) for the K-type thermocouples (0.005 inch diameter, Omega Engineering, Stamford, CT, USA). The thermocouples were connected to a data acquisition system (model PCI 4351, National Instruments, Austin, TX, USA) that converted the analog signal to a digital one. To control the temperatures for denaturation at 94 °C, annealing at 56 °C and extension at 72 °C, a computer received the temperature signal through a PCI-4351 interface (National Instruments) and determined the power input to the heater using a homemade fuzzy proportional/integral/derivative (PID) control algorithm that was programmed with LabVIEW 8.0 (National Instruments).

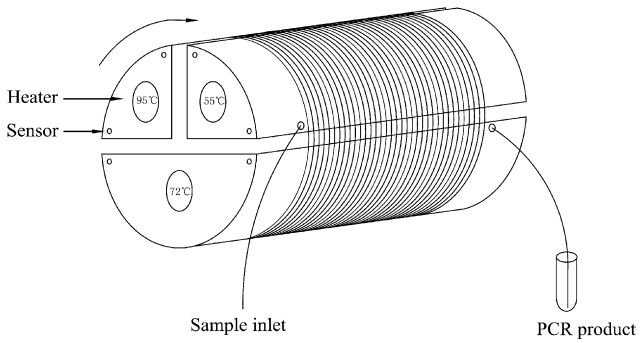

Fig. 1. Schematic diagram of continuous-flow PCR microfluidics.

A 5.2-m long transparent polytetrafluoroethylene (PTFE) capillary (0.5 mm i.d./0.9 mm o.d., Wuxi Xiangjian Tetrafluoroethylene Product, Wuxi, China) includes the inlet for samples injection and the outlet for product collection. It enters the cylinder through a hole (1.5 mm diameter) crossing the annealing and denaturation zones, providing an initial denaturation step. Then the capillary is wound 35 cycles in the spiral channel (1.1 mm width and 1.1 mm depth). The capillary exits the cylinder through a hole (1.5 mm diameter) in the elongation region, providing an additional extension on the 35th cycle. The inlet and outlet lengths of the capillary both were approximately 0.35 m. When the PCR cycles were increased to 45, the length of PTFE capillary was changed to 6.5 m. This device can perform up to 60 cycles.

Reagents and samples

PCR reagents, 10× Taq DNA polymerase buffer (500 mM KCl and 100 mM Tris–HCl, pH 8.8), $MgCl_2$ solution (25 mM), and thermostable Taq polymerase (5 U μl^{-1}) were purchased from Bio Basic (BBI, Ontario, Canada). Deoxynucleotide triphosphates (dNTPs, 10 mM each of dATP, dGTP, dCTP, and dTTP) and PCR primers [11] (Table 1) were obtained from Shanghai Sangon Biological Engineering & Technology Services (SSBE, Shanghai, China). The doubly deionized H_2O (ddH_2O) was provided by Tiangen Biotech (Beijing, China). GM soybeans were gifts from Guangdong Entry–Exit Inspection and Quarantine Bureau (Guangzhou, China), and the genomic DNA of soybean was extracted by using a plant genomic DNA extraction kit (Win Honor Bioscience [South], Guangzhou, China).

Bovine serum albumin (BSA, fraction V, purity ⩾ 98%, biotechnology grade, cat. no. 735094), which was used to dynamically coat the inner surface to decrease the surface adsorption [25], was purchased from Roche Diagnostics (Mannheim, Germany). Sodium hypochlorite solution, which was used to remove the residual DNA from the microchannel after each of the amplifications, was obtained from Guanghua Chemical Factory (Guangzhou, China). GoldView dye and SYBR Green I were purchased from SBS Genetech (Beijing, China). The DNA markers, which contain 2000-, 1000-, 750-, 500-, 250-, and 100-bp DNA fragments, were obtained from Win Honor Bioscience (South).

Continuous-flow PCR amplification

For the continuous-flow PCR amplification, 25 μl of PCR mixture consists of 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, the primer pair (0.5 μM each), 5 ng μl^{-1} soybean genomic DNA, 0.05 U μl^{-1} Taq DNA polymerase, and 0.025% (m/V) BSA. The PCR mixture was introduced into the capillary from the inlet and compelled by the precision syringe pump (cat. no. CZ-74901-15, Cole–Parmer