MICROFABRICATED FLOW-THROUGH PCR DEVICE FOR IN SITU GENE ANALYSIS IN EXTREME ENVIRONMENTS

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ABSTRACT
A microfabricated polymerase chain reaction (PCR) device for in situ microbiological study was developed and evaluated as the main components of totally integrated in situ gene analysis systems. The device consists of a PDMS chip containing a microchannel and a glass chip with integrated heaters and temperature sensors. The device was activated by a miniature pump and valve system to supply samples and PCR mixture. To evaluate the performance of the device, 580 and 1460 bp DNA fragments were targeted and successfully amplified within 30 and 60 min, respectively, from extracted E. coli whole genome or living cells directly.

Keywords: flow-through PCR, PDMS, continuous amplification, in situ analysis

1. INTRODUCTION
Within the last decade, importance of understanding the microorganisms inhabiting in extreme environments (extremophiles) such as deep-sea, deep-subsurface and under glacial lakes are widely recognized [1, 2]. Although molecular biological technique based on gene analysis is one of major research methods for environmental microbiology because of its high sensitivity, the technique has disadvantages such as time consuming and non-real time in principle. To obtain more information about environmental microbiology in detail, in situ gene analysis systems are strongly required. Especially for the study of extremophiles, totally automated and miniaturized analysis systems are essential because of human inaccessibility to such environments.

The flow through (or continuous flow) PCR is one of the useful alternatives to perform high-throughput gene amplification with minimum thermal masses [3]. During flow-through in a microchannel, temperature in reaction mixture is regulated on the each isothermal temperature zone instead of heating and cooling static sample. Some microfabricated devices for flow-through PCR were already reported and evaluated [4, 5]. However, integrated devices including pumps and valves towards in situ application have not reported yet. Furthermore, to use in extreme environments, miniaturization of whole system is also important because of limited payload capacity of small carrier such as ROVs (Remotely Operated Vehicles) or AUVs (Autonomous Underwater Vehicles).
this study, a microfabricated flow-through PCR device was developed aim at completion of totally integrated in situ gene analysis system. Miniature pump and valve systems are employed supposing stand-alone usage. To demonstrate capability for continuous amplification of varying natural samples, flow-through PCR was performed with real growing microbes.

2. DESIGNS AND FABRICATION

The device consists of a PDMS chip, where folded microchannels are fabricated to introduce reaction fluid, and a glass substrate integrated with heaters and temperature sensors to obtain three temperature zones (Fig. 1). To achieve transparent format for optical detection and observation, ITO (Indium Tin Oxide) was deposited and patterned on the bottom side of the glass substrate (50.8 mm x 76.2 mm). A thin layer of Pt (Platinum) was patterned as temperature sensors on the topside. Two pairs of a heater and sensor were patterned for each temperature zone to obtain uniform temperature distribution.

A PDMS with approximately 3 m-long (100 μm wide and deep) folded microchannel was bonded on a thin (0.12 mm) cover glass with spin coated PDMS layer. Since the PDMS chip is not directly bonded onto the glass chip, the glass chip can be used repeatedly by replacing the PDMS chip without any risk of chip-to-chip cross-contamination. The microchannel was designed to perform 30 cycles of PCR including prolonged the initial denaturation and the final elongation step.

The device was equipped with PC-controlled miniature pumps and valves (The Lee Company, USA) to supply and propel samples to be analyzed and reagent for PCR into the PDMS microchannel (Fig. 2).
Before the reaction, MPC (2-methacryloyloxyethyl phosphorylcholine) based polymer [6] was coated onto the PDMS channel surface to prevent adsorption of DNA polymerase molecules onto it.

3. EXPERIMENTAL

To evaluate the performance of the present device, PCR experiments were carried out with two kinds of PCR primer pairs (341f-926r and 27f-1492r), which are commonly used to amplify approximately 580 and 1460 bp DNA fragments of bacterial 16S rRNA gene for detection and characterization of eubacteria. As templates for flow-through PCR, extracted and purified E. coli genomic DNA or their living cells were used as model microbe. For amplification from E. coli living cells, 5% of Tween 20 (ICN Biomed. Inc. USA) was added into reaction mixture to ensure cell lysis during initial denaturation step. Addition of Tween 20 is also effective to reduce adsorption of reaction mixture components or bacterial cells onto the surface of pumps, valves and tubing.

For continuous monitoring of their growth, E. coli seeded culture medium was supplied sequentially during flow-through PCR operation. The initial cell concentration was 10^6 cells/ml and the culture medium was kept at 37 °C during continuous amplification experiment. Each temperature zone of flow-through PCR device was set at 95 °C for denaturation, 50 °C for annealing and 72 °C for elongation, respectively. Total flow rate was controlled by two miniature pumps (Flow rate ratio is 1:1).

4. RESULTS AND DISCUSSION

Both 580 and 1460 bp DNA fragments were successfully amplified within 30 and 60 min from E. coli whole genome with comparable or higher efficiency than that of the conventional thermalcycler (Fig.3). Specificity of amplification for 580 bp fragments was improved and long extra products were not amplified because of rapid thermal cycling compared with the conventional one.

Growth of E. coli was continuously monitored by applying the culture medium sequentially to the flow-through PCR device (Fig. 4). 580 bp PCR products appeared after 240 min from the beginning of growth of E. coli (lane 6). During the next 80 min (lane 7, 8), amount of PCR products were increased and then saturated (lane 9 to 20). Concentrations of E. coli cells were less than 10^6 cells/ml from 240 to 320 min (lane 6 to 8). This result implies that the flow-through PCR device have enough high sensitivity for in situ applications. For example, inhabitation of up to 10^7 - 10^8 /ml of microbes are estimated in deep-sea hydrothermal environments [7,8]. By operating the microfabricated flow-through PCR devices with appropriate primer pairs, detection of various kinds of microbes or functional genes becomes possible during operation of ROVs and AUVs. During 800 min of continuous amplification, any clogging of microchannel was observed and temperature sensors were also kept stable. According to this stability of the device, potential for application to long-term analysis is also suggested.
Fig. 3 Amplified DNA fragments with conventional thermalcycler (lane 1, 3) and the flow-through PCR device (lane 2, 4). Lane M: 200 bp DNA size marker.

Fig. 4 Result of continuous amplification with growing E. coli culture. Amplified products were collected every 40 min (Total 800 min operation). Lane M: 200 bp DNA size marker.

CONCLUSION
Flow-through PCR device that reported here is suitable for in situ usage because of simple temperature and fluid control. Furthermore, continuous in situ analysis is possible by applying various environmental samples to the device sequentially. Well-miniaturized gene analysis systems with flow through PCR device such as that reported here can be applied to extreme environments as a robust "micro Total Analysis Systems".

REFERENCES