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Microfabricated flow-through device for DNA amplification—towards in situ gene analysis

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Abstract

This study presents a microfabricated device for polymerase chain reaction (PCR) with a flow-through manner for use in environmental microbiology. The device was developed utilizing photolithography and softlithography techniques and evaluated as one component of a totally integrated in situ gene analysis system. The developed device was composed of a glass-based "temperature control chip" and polydimethylsiloxane (PDMS)-based "microchannel chip". For the temperature control chip, six heaters made from indium tin oxide (ITO) are placed on a glass substrate to define three uniform temperature zones for flow-through PCR. On the each heater, a platinum (Pt) line was placed as a temperature sensor. The PDMS microchannel structure was fabricated by using a molding method with a negatively patterned mold master. The width and depth of folded microchannel was 100 μm and total length for 30 cycles of flow-through PCR was approximately 3.0 m. With the flow-through PCR device, 580 and 1450 bp (base pairs) of DNA fragments were successfully amplified from *Escherichia coli* genomic DNA and directly from untreated cells.

Keywords: Flow-through PCR; Microfabrication; PDMS

1. Introduction

In recent years, gene-based analysis with molecular biological techniques has become essential for most research field relating to any living organisms. In particular, polymerase chain reaction (PCR) and its various derivatives are the most important techniques to amplify targeted DNA fragments from temperate DNA, or complementary DNA (cDNA) samples. To perform PCR, appropriate temperature cycling is necessary for denaturation (e.g. 96 °C) of double-stranded DNA, annealing of oligonucleotide primer pairs (e.g. 50 °C) and enzymatic elongation of new DNA strands catalyzed by DNA polymerase (e.g. 72 °C). With properly designed primer pairs, the desired region of DNA fragments can be amplified with high selectivity and sensitivity by performing 20–30 times temperature cycling. However, conventional techniques of performing PCR require much time and manpower. Thus, automation of routine work such as sample preparation (e.g. DNA extraction and purification), PCR and analysis (e.g. electrophoresis) is strongly demanded. Especially for the field of environmental microbiological study, totally automated and miniaturized devices for in situ gene analysis are essential to analyze unique ecosystems in extreme environments such as deep-sea [1], deep subsurface [2] and under-glacial lakes [3] as challenging targets. In most of the cases, sampling-based methods have been employed to this day, but some serious problems are caused by contamination or time lag between sampling and analysis with these methods. Well-automated and miniaturized in situ gene analysis systems can solve such problems and completely novel knowledge will be obtained about the frontiers of life.

These years, many examples of miniaturized PCR devices are reported in the field of research called "micro-total analysis systems (Micro-TAS)" or "Lab-on-a-chip" [4–6]. One of the unique alternative methods for PCR is flow-through (continuous-flow) PCR [7] and some miniaturized devices for flow-through PCR are also reported [8–10]. A system for flow-through PCR is composed of several constant and uniform temperature zones and folded or looped long capillary to carry PCR reagents on each temperature zones instead of heat blocks and reaction tubes (Fig. 1). This method was primarily proposed for high-speed PCR because its small thermal mass makes it capable of rapid temperature cycles compared with a conventional method [7]. Furthermore, continuous amplification for variable samples is also possible by supplying samples sequentially [11] and this feature is

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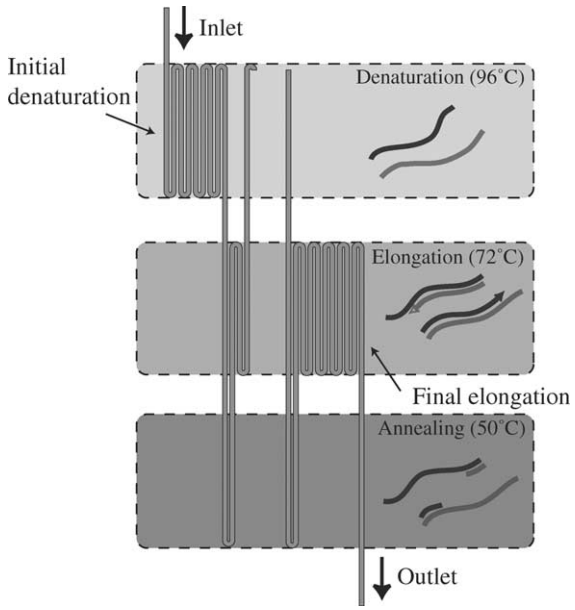

Fig. 1. Conceptual drawing of the flow-through (continuous-flow) PCR. States of DNA molecules at each temperature zone are illustrated.

very advantageous for in situ analysis of living microbes in nature. By analyzing components and dynamic change of them on the site with totally automated and miniaturized gene analysis system, many new characteristics of microbial ecosystems will be revealed.

Some microfabricated devices were developed to analyze inorganic components such as nitrate [12] or nitrate, silicate and phosphate [13] in oceanic environments and then the device for environmental microbiology is under development [14]. In this study, a miniaturized flow-through PCR device was developed and evaluated as a core part of totally integrated systems towards in situ gene analysis of microbial ecosystems at extreme environments. A transparent silicone elastomer (polydimethylsiloxane (PDMS)) was used to obtain microchannel structures. By employing molding-based softlithography technique, PDMS microchannel structure can be created with high reproducibility and low cost [15,16]. The temperature control chip can be used repeatedly without any risk of contamination by changing only the PDMS microchannel chip for various flow-through PCR operations. *Escherichia coli* cells and extracted and purified genomic DNA was employed for evaluation of the developed flow-through PCR device.

2. Materials and methods

2.1. Fabrication and calibration of temperature control chip

Design of the temperature control chip is shown in Fig. 2. Platinum (Pt) and indium tin oxide (ITO) layers on each face of a glass substrate (50.8 mm × 76.2 mm) were photo-patterned using conventional wet etching processes to make the temperature sensors and transparent heaters, respectively (Fig. 3 (A1–3)). The surface of temperature sensors was covered with a SiO_2-based thin layer (OCD T-7, TOKYO OHKA KOGYO Co. Ltd., Japan) for electrical insulation and protection from physical damaging (Fig. 3 (A4)). After connecting electrical lines for the temperature control chip, temperature sensors that work as resistive temperature detectors (RTDs) were calibrated and the temperature–resistance conversion curves were obtained.

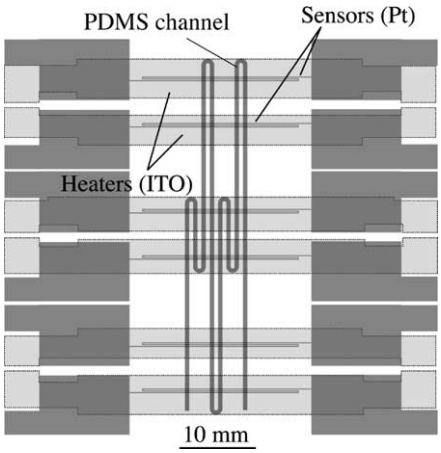

Fig. 2. Schematic view of six pairs of heater (ITO) and temperature sensor (Pt). A folded PDMS microchannel is also shown.

2.2. Softlithography

The PDMS chips containing the microchannel structure for flow-through PCR were fabricated using softlithography technique. An ultra thick negative photoresist (SU-8; Microchem, USA) was photo-patterned onto a silicon wafer to make a negative master of the microchannel. The thickness of SU-8 was 100 μm to obtain a 100-μm-deep microchannel. For PDMS release, the wafer was coated with fluorocarbon layer polymerized by CHF_3 plasma in a reactive ion etching (RIE) machine (Fig. 3 (B1 and B2)). A prepolymer of PDMS was poured onto the wafer and cured at 75 °C for 120 min (Fig. 3 (B3)). After peeling off the cured PDMS with transferred microchannel structure (Fig. 3 (B4)), holes for inlet and outlet of reagents were drilled through manually. The PDMS channel chip was permanently bonded with a thin PDMS layer that was spin coated on a glass plate (0.12–0.17 mm thick) by exposing to O_2 plasma in a RIE machine to accomplish a microchannel chip (B5). In PDMS microchannel with total length of 3033 mm, 30 temperature cycles could be performed including a prolonged initial denaturation and final elongation steps. The microchannel was designed to execute a residence time ratio of 2:2:3 for the reaction mixture in each temperature zone for denaturation, annealing and elongation, respectively.

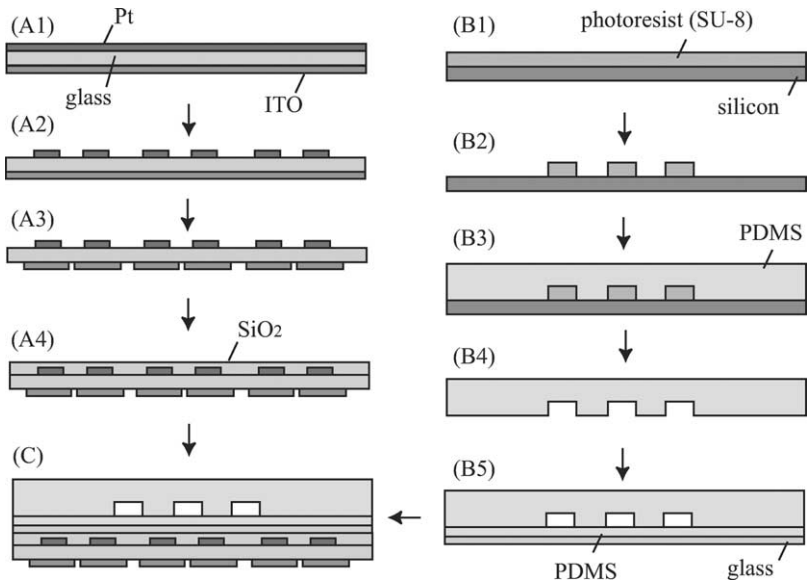

Fig. 3. The microfabrication process of the flow-through PCR device. A1–4: Photo-fabrication of the temperature control chip. B1–5: Fabrication of PDMS channel chip using a molding method. C: Assembly of the device.

2.3. Assembly and surface treatment

The temperature control chip and the PDMS microchannel chip were assembled using a PMMA housing and fixed onto a metal frame (Fig. 3(C)). Holes for inlet and outlet were drilled into the PMMA housing.

The surface of the PDMS channel was coated with 2-methacryloyloxyethyl phosphorylcholine (MPC)-based polymer [17] with a silane coupler to prevent adsorption of protein molecules (e.g. DNA polymerase) onto it. Immediately after the permanent bonding process, the PDMS channel was filled with the polymer solution and kept still for 30 min. Next, the polymer solution was removed and the PDMS channel was briefly rinsed with pure ethanol. Finally, the PDMS channel chip was cured at 100 °C for 1 h to polymerize the MPC polymer.

2.4. PCR chemistry and control experiments

The reaction mixture for flow-through PCR was prepared as follows. The mixture contained 50 mM of each deoxynucleosidetriphosphate, 1.5 μM $MgCl_2$, 0.05 U/μl *Taq* DNA polymerase (TAKARA *Taq*: TAKARA BIO Inc., Japan) and 400 ng/ml of *E. coli* genomic DNA. For direct PCR amplification, liquid cultured *E. coli* cells (7.7×10^8 cells/ml in 30% LB culture medium, NACALAI TESQUE Inc., Japan) were used as source of PCR templates without any purification steps. The cultured medium containing *E. coli* cells was added to make up one half of the total amount of PCR mixture. 100 nM of each oligonucleotide primer pairs specific to bacterial 16S rRNA gene fragment Eubac27F–Eubac1492R [18] and 341F–926R [19] were used. The primer sequences were as follows: Eubac27F (5′-AGA GTT TGA TCC TGG CTC AG), Eubac1492R (5′-GGT TAC CTT GTT ACG ACT T), 341F (5′-CCT ACG GGA GGC AGC AG) and 926R (5′-CCG TCA ATT C(A/C)T TTG AGT TT).

The temperature for each temperature zone was set at 96 °C for denaturation, 50 °C for primer annealing and 72 °C for elongation.

Control PCR amplification was carried out with a conventional thermal cycler (TAKARA PCR Thermal Cycler PERSONAL: TAKARA BIO Inc., Japan). Reaction conditions were as follows: 30 cycles of 30 s at 96 °C for denaturing, 30 s at 50 °C for annealing and 45 s at 72 °C for elongation. Ninety seconds for denaturation (totally 120 s) and 255 s for elongation (totally 300 s) was added to the initial and the final steps of PCR, respectively. Total time to complete 30 cycles of PCR was approximately 85 min for control amplification.

PCR amplified fragments were analyzed after fractionation by agarose slab gel electrophoresis. Ten microliters of each sample were loaded onto 1% of agarose gel and electrophoresed in 1× tris acetate EDTA (TAE) buffer. After electrophoresis, the gel was stained with ethidium bromide for visualization on a UV transilluminator (254 nm).

3. Result and discussion

Three well-defined temperature zones were obtained with six pairs of ITO heaters and Pt temperature sensors. Pt temperature sensors as RTDs showed highly linear relationships between the value of resistance and real temperature. The microfabricated flow-through PCR device was completed by assembling the temperature control chip with electrical line

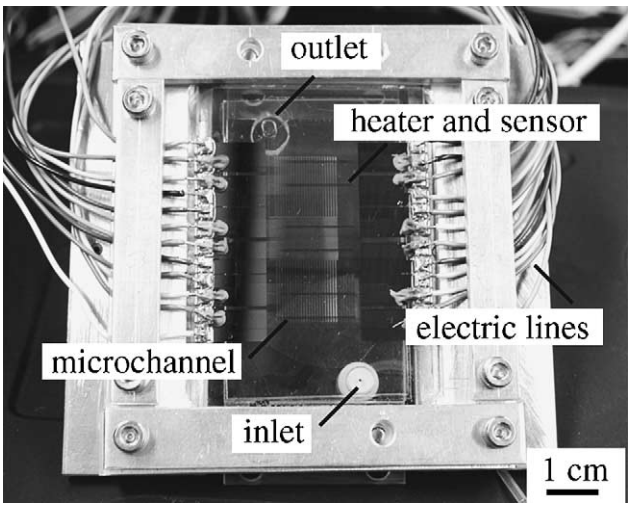

Fig. 4. A photograph of the flow-through PCR device.

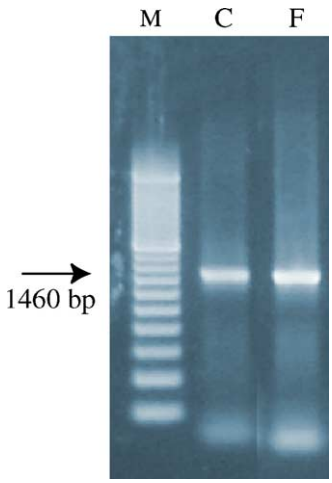

Fig. 6. Result of flow-through PCR to amplify 1460 bp DNA fragments (lane F: flow rate is 30 μl/h). Lane M: DNA size marker at every 200 bp. Lane C: Control PCR with a conventional thermalcycler.

and the PDMS microchannel chip by using a chip housing as shown in Fig. 4.

Adsorption of DNA polymerase onto the intact surface of the PDMS microchannels was apparently terminated by MPC-based polymer coating. There was no amplification product without coating due to the adsorption problem (data not shown) and thus, all of PDMS microchannels used in this study were coated with MPC-based polymer.

The 580 bp of 16S rRNA gene fragments were successfully amplified from extracted and purified *E. coli* genomic DNA (Fig. 5) PCR was performed with various flow rates (30–150 μl/h) continuously. Amplified products were collected every 30 μl from an outlet port. For each flow rate condition, two consecutive products were collected, because the first products contain the amplified fragments performed with preceding flow rate conditions. Yield of amplification performed at 30–60 (micro)l/h with flow-through PCR device was comparable to that with conventional thermalcycler. Although the amount of amplified fragment decreased with higher flow rates, the improvement of specificity was certified with disappearances of undesired longer DNA fragments caused by non-specific amplification. After operation at flow rate of 150 μl/h, the flow rate was decremented to 30 (micro)l to confirm the reproducibility of PCR. After reduction of the flow rate, the amplification yield was recovered. The 580 bp fragment amplified in this study is one of a commonly used region of gene for density gradient gel electrophoresis (DGGE) analysis to ascertain diversity and structure of microbial communities [20]. Thus, this result implies the flow-through PCR can be applied to high throughput characterization of microbial communities with integration of appropriate functions for analysis steps such as capillary electrophoresis [5,6,21].

Relatively longer (1460 bp) DNA fragments of bacterial 16S rRNA gene, which can be used for gene-based identification of microbes, were also amplified successfully in 60 min (flow rate was 30 μl/h) (Fig. 6). The yield of PCR amplification was higher than that of conventional methods. Amplified DNA fragments can be also applied to 16S rRNA-restriction fragment length polymorphisms (RFLP) analysis of genetic diversity of microbial communities [22].

The 580 bp of 16S rRNA gene fragments were also amplified directly from *E. coli* cells (Fig. 7). The *E. coli* cells were lysed during denaturation step (96 °C) by adding detergent (Tween 20) to the PCR reagent. Approximately 3.9×10^8 cells were contained in 1 ml of PCR mixture for this experiment. The time required for amplification was same as

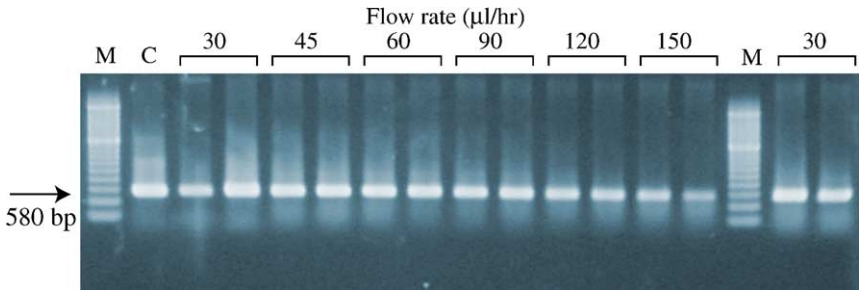

Fig. 5. Gel photograph of 580 bp DNA fragments amplified by conventional thermalcycler (lane C) and the flow-through PCR device (lanes 2–14). The flow rate for each flow-through PCR operation is also shown. Lane M: DNA size marker at every 200 bp.

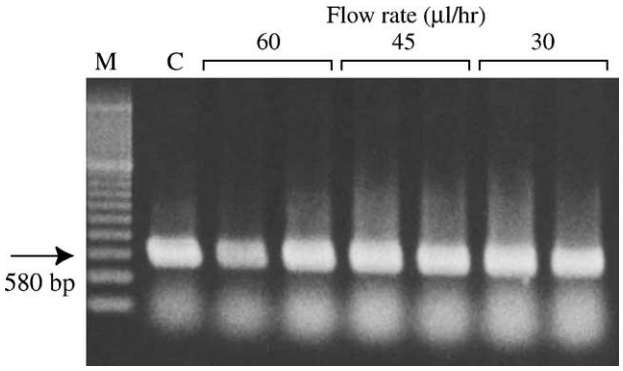

Fig. 7. Result of PCR amplification of 580 bp DNA fragments directly from *E. coli* cells. Lane C: Amplified DNA fragment with conventional thermalcycler.

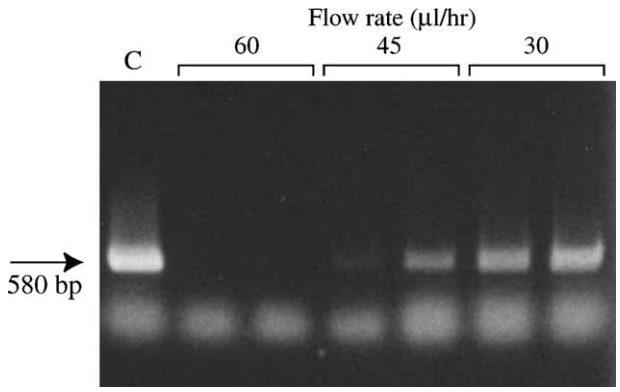

Fig. 8. Result of PCR amplification of 580 bp DNA fragments directly from diluted *E. coli* cells. Lane C: amplified DNA fragment with conventional thermalcycler. In the case of 60 μl/h, amplified fragments were not observed.

the PCR with the extracted and purified genomic DNA as a template (within 30 min). Direct amplification is very advantageous for high throughput analysis of relatively fragile microbes such as *E. coli*. For tougher microbes such as spore forming microbes, integration of cell lysis [23] or destruction function [24] to flow-through PCR device may be necessary.

The direct PCR was also carried out by applying diluted *E. coli* cells (1.3×10^6 cells/ml of PCR mixture) to the flow-through PCR to estimate the detection limit (Fig. 8). As a result, detectable amount of product was not obtained in the case of the flow rate at 60 (micro)l/h and the amplification yields increased with smaller flow rates (45 or 30 (micro)l/hr). According to this result, the flow-through PCR device we developed can be applied to real environments containing targeted microbes on the order of 10^6 cells/ml. By performing flow-through PCR with smaller flow rates, the detection limit will be improved.

4. Conclusion

The flow-through PCR device developed in this study is suitable for in situ usage because of the simplicity of temperature and fluid control. Integration of miniaturized pumps to the device is in progress now [25]. By improving the detection limits, the device can be applied to operation in real environments that harbor fewer microbes than the order of 10^6 cells/ml. Furthermore, continuous analysis is possible by applying various environmental samples to the device sequentially. For example, detection of various peculiar microbes or functional genes becomes possible in aquatic extreme environments such as deep-sea or under-glacial lakes using remotely operated vehicles (ROVs) and autonomous underwater vehicles (AUVs) equipped with the flow-through PCR device. Thoroughly miniaturized and automated devices can be penetrated into boreholes drilled in sea floor, enabling surveys of the abundance and diversity of microbial ecosystems widely distributed in deep subsurface environments forming the frontier of life on Earth.

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References

[1] C.D. Winn, D.M. Karl, Nature 320 (1986) 744.
[2] T.O. Stevens, J.P. McKinley, Science 270 (1995) 450.
[3] S.S. Abyzov, I.N. Mitskevich, M.N. Poglazova, Microbiology 67 (1998) 451.
[4] M.A. Burns, B.N. Jhonson, S.N. Brahmasandra, K. Handique, J.R. Webster, M. Krishnan, T.S. Sammarco, P.M. Man, D. Jones, D. Heldsinger, C.H. Mastrangelo, D.T. Burke, Science 282 (1998) 484.
[5] L.C. Waters, S.C. Jacobson, N. Kroutchinina, J. Khandurina, R.S. Foote, J.M. Ramsey, Anal. Chem. 70 (1998) 158.
[6] J.W. Hong, T. Fujii, M. Seki, T. Yamamoto, I. Endo, Electrophoresis 22 (2001) 328.
[7] H. Nakano, K. Matsuda, M. Yohda, T. Nagamune, I. Endo, T. Yamane, Biosci. Biotech. Biochem. 58 (1994) 349.
[8] M.U. Kopp, A.J. de Mello, A. Manz, Science 280 (1998) 1046.
[9] I. Schneegab, R. Bräutigam, J.M. Köhler, Lab on a Chip 1 (2001) 42.
[10] P.J. Obeid, T.K. Christopoulos, H.J. Crabtree, C.J. Backhouse, Anal. Chem. 75 (2003) 288.
[11] M. Curcio, J. Roeraade, Anal. Chem. 75 (2003) 1.
[12] H.W. Jannasch, K.S. Johnson, C.M. Sakamoto, Anal. Chem. 66 (1994) 3352.
[13] D. Thouron, R. Vuillemin, X. Philippon, A. Lourenço, C. Provost, A. Cruzado, V. Garçon, Anal. Chem. 75 (2003) 2601.
[14] T. Fukuba, T. Naganuma, T. Fujii, in: Proceedings of the 2002 International Symposium on Underwater Technology, 2002, p. 101.
[15] E. Delamarche, A. Bernard, H. Achmid, B. Michel, H. Biebuyck, Science 276 (1997) 779.
[16] D.C. Duffy, J.C. McDonald, O.J.A. Schueller, G.M. Whitesides, Anal. Chem. 70 (1998) 4974.
[17] K. Ishihara, H. Nomura, T. Mihara, K. Kurita, Y. Iwasaki, N. Nakabayashi, J. Biomed. Mater. Res. 39 (1998) 323.
[18] E.F. DeLong, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 5685.

[19] G. Muyzer, S. Hottentrager, A. Teske, C. Wawer, Molecular Microbial Ecology Manual, Kluwer Academic Publishing, Dordrecht, 1996, p. 3.4.4.1
[20] G. Muyzer, Curr. Opin. Microbiol. 2 (1999) 317.
[21] S.H. Chen, Y.H. Lin, L.Y. Wang, C.C. Lin, G.B. Lee, Anal. Chem. 74 (2002) 5146.
[22] O.K. Radjasa, H. Urakawa, K. Kita-Tsukamoto, K. Ohwada, Mar. Biotechnol. 3 (2001) 454.
[23] S.W. Lee, Y.C. Tai, Sens. Actuators 73 (1999) 74.
[24] P. Belgrader, D. Hansford, G.T.A. Kovacs, K. Venkateswaran, R. Mariella, F. Milanovich, S. Nasarabadi, M. Okuzumi, F. Pourahmadi, M.A. Northrup, Anal. Chem. 71 (1999) 4232.
[25] T. Fukuba, T. Naganuma, T. Fujii, in: proceedings of the Micro-TAS 2003 Symposium, 2003, p. 725.