DEVELOPMENT OF NEW BIOCHEMICAL IC CHIP-SET FOR REAL-TIME PCR

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ABSTRACT

We have developed new biochemical IC chip-set which realizes real-time PCR in the finger top size. Unlike conventional μ-TAS approach, our chip-set has great versatility and portability since all chips are designed in standardized micro-architecture and the fabrication process can be combined with conventional MEMS processes. Combining this chip-set with existing Biochemical IC chip-set family, researchers can easily develop order-made palm-top system for proteomics research, on-site diagnosis, and tailor-made medicine.

INTRODUCTION

In recent years, the infection of SARS, Bird Flu and other infectious diseases have been spreading over the world. To prevent the pandemic of serious diseases, rapid detection and on-site analysis of the infection is highly required. The key technology for rapid diagnosis of infection is real-time PCR. Real-time PCR is a technique which is used to amplify and simultaneously quantify the target DNA. Since it enables DNA amplification and simultaneous quantification by comparing the fluorescence signal from the product to a standard curve calibrated by PCR of a known DNA, we can diagnose more rapidly compared to conventional PCR. There is a problem, however, that real-time PCR apparatuses are so large to carry out on-site diagnosis. Even the recently advancing lab-on-chip and μ-TAS technologies cannot be applied directly to the on-site diagnosis, because they only focus on the miniaturization of specific function –e.g. reaction and detection [1], and not the whole device. Therefore, miniaturization of the whole system for real-time PCR is strongly needed.

BIOCHEMICAL IC CHIPS

To overcome the above issue, we have been proposing and developing Biochemical IC chips. The Biochemical IC, which is proposed by Ikuta [2-4] in 1993, is a group of standardized micro chemical device with various functions for biological experiments. Each chip has a specific function, and arbitrary micro-systems for biological analysis can be realized by assembling these chips (Figure 1). To date, we have developed chips for reaction [5], concentration [6], pumping [7], homogenization [8], cell-free protein synthesis [9], protein analysis (protein separation and collection) etc.

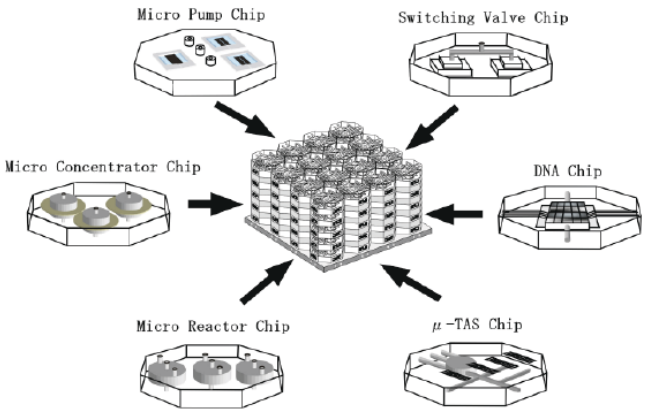


Figure 1: Module Concept of Biochemical IC

Recently we have developed "μ-Total Protein Analysis System" by combining these chips, and we can do the experiment from cell homogenization to collection of cell protein in the finger top [10]. These applications are the necessary techniques for the post-genome research such as proteomics research.

In this paper, development of a new Biochemical IC chip-set for real-time PCR is reported. Using the chip-set, accurate temperature control over an hour with high reproducibility was verified, and we have successfully amplified specific DNA and simultaneously quantified the products by fluorescence intensity with a CCD camera.

CONCEPT OF REAL-TIME PCR CHIP-SET

The following is the required specifications to design new Biochemical IC chip-set for real-time PCR.

(1) A precise and high speed temperature control which can realize real-time PCR thermal cycling
(2) Highly compatible and extensible structure suitable for module concept
(3) Detection of the fluorescence at multiple points in the reactor chip to realize a number of real-time PCR reactions in parallel.
(4) Fluorescence excitation and detection

Here is the schematic of Real-time PCR chip-set concept (Figure 2).

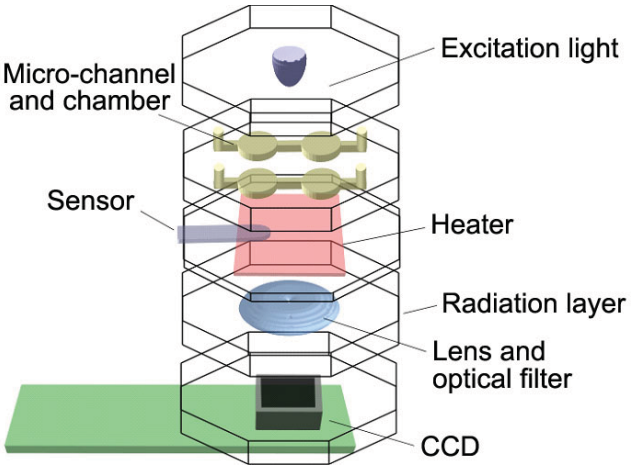


Figure 2: Concept of Real-time PCR chip-set

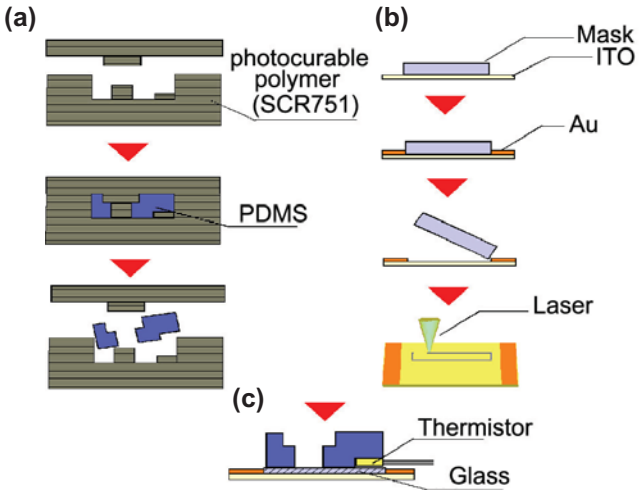


Figure 3: Fabrication process of the real-time PCR chip-set
(a) Fabrication of the micro-reactor
(b) Fabrication of the temperature control module
(c) Assembly of the two parts by PDMS bonding

We adopt a transparent conductive film since it can transmit the excitation light and fluorescence. Besides, it can work as a heater at the same time. A micro-reactor that has micro-channels and chambers is used to fulfill the 2nd and the 3rd specification stated above. To realize the 4th specification, Blue LED is set above the micro-reactor as an excitation light source. The fluorescence from the marker specific to double strand DNA in the sample is focused by an objective lens, and detected by a CCD sensor through a band-pass optical filter.

FABRICATION OF THE REAL-TIME PCR CHIP-SET

The micro-reactor must have PCR compatibility and transparency, and the temperature control module should be capable of controlling PCR thermal cycling for a number of times. We adopted PDMS as a material of the micro-reactor since it is widely used in biochemical and medical devices, and the more, it has fine transparency. The fabrication process of the micro-reactor and the temperature control module are described below (Figure 3).

Fabrication process of the micro-reactor (Figure 3 (a))

(1) Fabricate molds made of photocurable polymer (SCR751, D-MEC) by microstereolithography and heat it for 12 hours at 200℃, then perform fluorine coating
(dip CTX-109AE (ASAHI GLASS) diluted by CT-Solv. 100E (ASAHI GLASS), and heat for 10 min at 80℃, then dip again and heat for 1 hour at 180℃)
(2) Mix PDMS (SYLGARD 184 SILICON ELASTOMER, TORAY/DOW CORNING) by BASE: CURING AGENT =10:1 and inject the premix into the mold
(3) Heat for 8 hours at 80℃ after removing bubbles *in vacuo*

Fabrication of the temperature control module (Figure 3 (b), (c))

(1) Cut the ITO (Indium tin oxide coated PET sheet, 35Ω/sq surface resistivity, Sigma-Aldrich.Inc) to the size of 6(mm)×13(mm)
(2) Place a stencil mask on the ITO to pattern the electrodes
(3) Fabricate a pair of electrodes by sputtering Au on the ITO/PET substrate
(4) Remove the mask
(5) Pattern the ITO layer by excimer laser (the line width is about 25 μm)

Assembly of the micro-reactor and the temperature control module

(1) Coat premixed PDMS on the surface of a cover glass (the thickness is about 0.15 (mm)) and combined with the micro-reactor by heating at 80℃ for 8 hours
(2) Attach the lower surface of the cover glass and the ITO using PDMS
(3) Remove the protective layer of the thermistor (104JT, ISHIZUKA ELECTORONICS) to improve the thermal response. A fine enamel wire is used to connect the thermistor and the voltmeter to decrease the heat capacity of the thermistor module. The module is then inserted into a preformed space for the thermistor in the micro-reactor, and enclosed by PDMS.
(4) Insert (3) into a holder to complete the real-time PCR chip-set.

Prototype of real-time PCR chip-set

The Real-time PCR chip-set developed consists of a PCR chip and a spacer chip (Figure 4). PCR chip is an assembly of a holder chip, a temperature control module and a micro-reactor made of PDMS.

The temperature control module has an ITO/PET substrate as a heater and a thermistor for temperature measurement. The ITO allows DNA amplification, irradiation of the excitation light, and real-time detection of

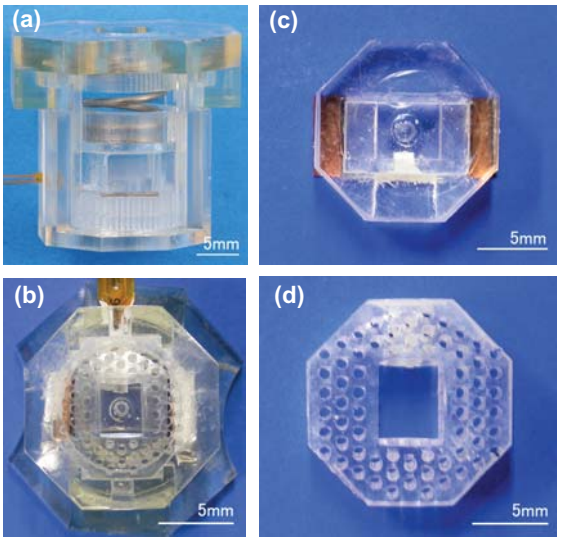


Figure 4: Fabricated Real-time PCR chip-set
(a) Assembly in a holder unit (side view)
(b) Assembly in a holder unit (top view)
(c) PCR chip (d) Spacer chip

fluorescence simultaneously due to its transparency. In addition, since we can observe from its top or bottom, there is a lot of flexibility to design optical system and it is easier to miniaturize the whole system.

TEMPERATURE CONTROL OF THE MICRO-REACTOR

The PCR chip and the spacer chip fabricated by the above process were set in the holder unit. We controlled PCR thermal cycle using this chip-set and measured the temperature on the ITO surface by thermograph. As a result, we confirmed that the temperature gradient is so steep for heat concentration at the center of the heater when we use the ITO formed on the PET uniformly. The temperature gradient becomes steeper as temperature rises. PCR needs to repeat the specified cycle for the DNA amplification, so this problem made it difficult to perform PCR in the micro-reactor and calibration was also difficult since a slight displacement causes big differences of temperature.

Improvement of the temperature distribution

To solve the problem, the ITO layer was patterned using excimer laser to make a uniform temperature distribution (Figure 5). As a result, we verified the uniform temperature distribution and successfully extended the PCR effective area.

In addition, we improved the structure of the spacer chip and modified the temperature distribution. The spacer chip fixes the temperature control module and the micro-reactor at a prescribed distance. Micro-holes are patterned on the spacer chip to correct the difference of heat conductivity in the temperature control module caused by the thermistor and homogenize the temperature distribution in the micro-reactor.

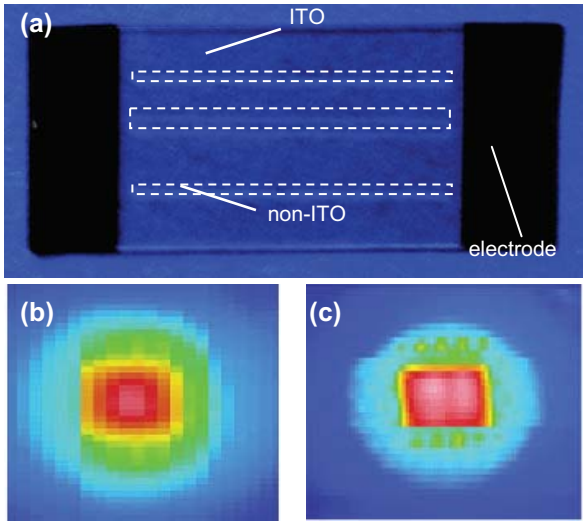


Figure 5: (a) Patterned ITO/PET substrate (b) Thermograph of the ITO/PET substrate before excimer laser patterning. (c) Thermograph of the ITO/PET substrate after excimer laser patterning

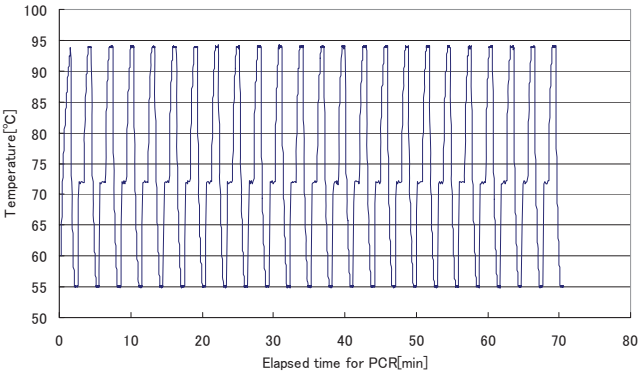


Figure 6: Temperature transition of the PCR cycle using the developed temperature control module

Verification of the uniform temperature distribution using the patterned ITO

To verify the temperature control using the chip-set, temperature cycles of real-time PCR (94℃・55℃・72℃) is demonstrated. Labview was used for PCR thermal cycle control. The results showed accurate temperature control over an hour with high reproducibility (Figure 6)

EXPERIMENTAL VERIFICATIONS OF REAL-TIME PCR FUNCTION

Experimental methods

We performed real-time PCR using the developed chip-set for the verification. We adopted the intercalater method for real-time PCR. The intercalater is a molecule that produces fluorescence when it combined with double-strand DNA. By adding the intercalater to the sample solution, the amount of the amplified unknown DNA can be estimated by comparing the measured fluorescence intensity and a standard curve calibrated by PCR of a known DNA.

Here, we performed 40 cycles of real-time PCR by assembling these chips in the holder unit, adding the intercalater to PCR sample, and injecting mineral oil over sample for preventing evaporation of sample.

And we obtained fluorescence images of the micro-reactor at each cycle (72℃・30sec) with a CCD camera to record the transition of fluorescence intensity. The excitation light was irradiated using a conventional fluorescence microscope instead of Blue LED. After the PCR reaction finished, the sample was taken out and electrophoresed using agarose gel. The fluorescence of the bands was observed using a transilluminator.

Experimental conditions

- Sample: 3(μl)
  intercalater (SYBR-Green Ⅱ, TAKARA BIO): F primer 2μM: R primer 2μM: template DNA 2g/l: sterile water =1.8:1:1:1:1.2
- Mineral oil: 3(μl)
  mineral oil for molecular biology, light oil (SIGMA)
- Fluorescence reagent for electrophoresis
  EnVISION DNA as Loading Buffer (Ameresco Inc)
- Excitation light / Florescence: Blue / Green

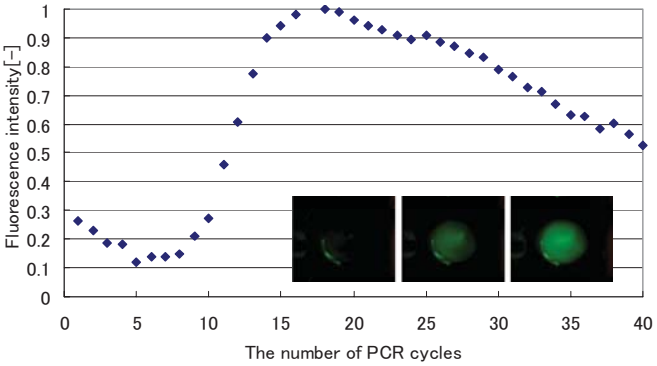
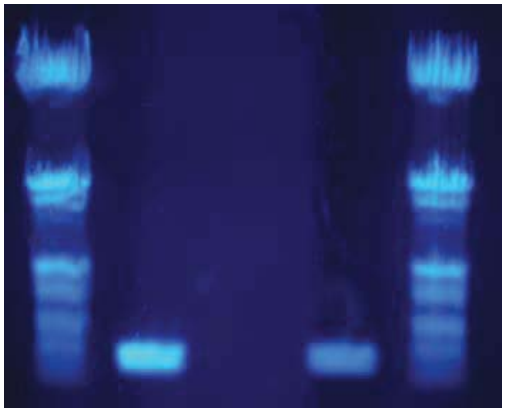


Figure 7: Transition of the fluorescence intensity during real-time PCR using the developed chip-set (the inset shows the CCD image of 8th, 12th and 16th cycle)



(a) (b) (c) (d) (e)

Figure 8: Electrophoresis of the products (a,e) marker (b) positive control (c) negative control (d) real-time PCR chip-set

PCR thermal cycle was as shown below.
94 ℃ (5sec)→[55 ℃ (30sec)→72 ℃ (45sec)→94 ℃ (30sec)]×40→72℃ (5min)

The result of real-time PCR

Figure 7 shows the transition of fluorescence signal from the micro-reactor. The fluorescence intensity increased in proportion to DNA amplification until it reached to the depletion of PCR resources at 16th cycle. Figure 8 shows the result of electrophoresis of the final products. Figure 8(b) shows positive control obtained by the conventional PCR apparatus and Figure 8(d) shows the result of the developed chip-set. The DNA band was detected at the same position as positive control. These results proved successful amplification of the target DNA and simultaneous quantification of the products in the new real-time PCR chip-set.

CONCLUSIONS

We have developed new Biochemical IC chip-set for real-time PCR. And we demonstrated successful amplification of the target DNA and simultaneous quantification of the products in the new real-time PCR chip-set. This chip-set has great versatility and portability since all chips are designed in standardized micro-architecture and the fabrication process can be combined with conventional MEMS processes. This chip-set together with other Biochemical IC family should be a powerful tool for on-site rapid detection of infections, tailor-made medicine and proteomics research.

REFERENCES

[1]S.-K.Hsiung and G.-B.Lee, “A controllable micro-lens structure for bio analytical applications,” Proc.of MEMS07, pp.763-766 (2007)

[2]K.Ikuta, K.Hirowatari and T.Ogata, “Tree dimensional micro integrated fluid system (MIFS) fabricated by stereolithography,” Proc.of MEMS 94, 1-6 (1994)

[3]K.Ikuta, T.Ogata, M.Tsuboi, S.Kojima, “Development of mass productive micro stereolithography (Mass-IH process),” Proc.of MEMS 96, pp.301-306 (1996)

[4]K.Ikuta, “3D micro integrated fluid system toward living LSI-Challenge for artificial cellular device-,”vArtificial Life V (MIT press), 17-24 (1997)

[5]K.Ikuta, S.Maruo, Y.Fukaya and T.Fujisawa, “Biochemical IC chip toward cell free DNA protein synthesis,” Proc.of MEMS 98,131-136 (1998)

[6]K.Ikuta, S.Maruo, T.Fujisawa and A.Yamada, “Micro Concentrator with Opto-sense Micro Reactor for Biochemical IC Chip Family -3D Composite Structure and Experimental Verification -,” Proc.of MEMS 99,376-381 (1999)

[7]K.Ikuta, T.Hasegawa, T.Adachi and S.Maruo, “Fluid drive chips containing multiple pumps and switching valve for biochemical IC chip family - Development of SMA drive 3D micro pumps and valves in leak-free polymer package -,” Proc.of MEMS 2000,739-744 (2000)

[8]K.Ikuta, A.Talahashi, K,Ikeda and S.Maruo, “Fully integrated micro biochemical laboratory using Biochemical IC Chips - Cell-free protein synthesis by using a built-in micropump chip -,” Proc.of MEMS 03,451-454 (2003)

[9]K.Ikuta, Y.Sasaki, H.Maegawa, and S.Maruo, “Biochemical IC Chip for pretreatment in biochemical experiments – Micro ultrasonic homogenizer chip made by hybrid microstereolithography -,” Proc. of MEMS 03,343-346 (2003)

[10]K.Ikuta, N.Satake, T.Ohashi and M.Shibata, “Finger-top Total Protein Analysis System based on New Biochemical IC Chips-,” Proc of MEMS 08, (2008)