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On-Chip Nanoliter-Volume Multiplex TaqMan Polymerase Chain Reaction from A Single Copy Based on Counting Fluorescence Released Microchambers

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A novel method for multiplex TaqMan PCR in nanoliter volumes on a highly integrated silicon microchamber array is described. Three different gene targets, related to β-actin, sex-determining region Y (SRY), and Rhesus D (RhD) were amplified and detected simultaneously on the same chip by using three different types of human genomic DNA as the templates. The lack of cross-contamination and carryover was shown using alternate dispensing of mineral oil-coated microchambers containing template and those without template. To confirm the specificity of our system to β-actin, SRY, and RhD genes, we employed the larger volume PCR samples to a commercial real-time PCR system, SmartCycler. The samples were cycled with the same sustaining temperatures as with the microchamber array. Instead of the conventional method of DNA quantification, counting the number of the fluorescence released microchambers in consequence to TaqMan PCR was employed to our chip. This simple method of observing the end point signal had provided a dynamic quantitative range. Stochastic amplification of 0.4 copies/reaction chamber was achieved. The microfabricated PCR chip demonstrated a rapid and highly sensitive response for simultaneous multiple-target detection, which is a promising step toward the development of a fully integrated device for the "lab-on-a-chip" DNA analysis.

Polymerase chain reaction (PCR)-based techniques have become the most important part of DNA diagnostic laboratories since its first introduction in 1985.[1] The discovery of this technology has earned its inventor, K. B. Mullis, a Nobel prize for his achievement,[2,3] which has opened new horizons for a limitless number of DNA-based research possibilities. Since then, qualitative PCR has been a well-established and straightforward technology, but the quantification of specific target DNA sequences in a complex sample has been a difficult task. A number of variations, caused by the manipulation of nucleic acids that may occur during sample preparation, storage, or the course of the reaction hampered accurate quantification. The exponential nature of the PCR amplification can significantly magnify even minor variations in reaction conditions. Normalizing the amount of PCR products of the specific template with respect to an internal reference template has been partly successful against these variations. Since the challenge of accurate DNA quantification stimulated many researchers, a great variety of protocols already exist for the utility of quantitative PCR.[4−6] However, these methods are nearly exclusively restricted to be applied for research purposes only because of two factors they have in common: they are difficult tasks and are costly to run.

To supply the demand for faster, more accurate, and more cost-effective PCR devices with a high-throughput capacity, three important properties have directed the development of the next generation of PCR systems: automation, standardization, and miniaturization. Recently, Yang et al.[7] reported a high-sensitivity PCR assay in polycarbonate plastic, disposable PCR microreactors. At a template concentration as low as 10 *Escherichia coli* cells (equivalent to 50 fg of genomic DNA), 221-bp product was successfully amplified within 30 min. Lee et al.[8] have described a microfabricated PCR device for simultaneous DNA amplification and electrochemical detection on gold or indium tin oxide (ITO) electrodes patterned on a glass substrate. A miniaturized flow-through PCR with different template types in a silicon chip thermocycler was also reported to have a minimum power consumption.[9] With the flow-through PCR device of Fukuba et al.,[10] 580 and 1450 bp of DNA fragments were successfully

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(1) Saiki, R. K.; Scharf, S.; Faloona, F.; Mullis, K. B.; Horn, G. T.; Erlich, H. A.; Arnheim, N. *Science* **1985**, *230*, 1350−1354.
(2) Mullis, K.; Faloona, F.; Scharf, S.; Saiki, R.; Horn, G.; Erlich, H. *Cold Spring Harbor Symp. Quantum Biol.* **1986**, *51* (Pt 1), 263−273.
(3) Mullis, K. B.; Faloona, F. A. *Methods Enzymol.* **1987**, *155*, 335−350.
(4) Irino, T.; Kitoh, T.; Koami, K.; Kashima, T.; Mukai, K.; Takeuchi, E.; Hongo, T.; Nakahata, T.; Schuster, S. M.; Osaka, M. *J. Mol. Diagn*. **2004**, *6*, 217−224.
(5) Hirt, C.; Schuler, F.; Dolken, L.; Schmidt, C. A.; Dolken, G. *Blood* **2004**, *104*, 904−905.
(6) Nurmi, J.; Wikman, T.; Karp, M.; Lovgren, T. *Anal. Chem*. **2002**, *74*, 3525−3532.
(7) Yang, J.; Liu, Y.; Rauch, C. B.; Stevens, R. L.; Liu, R. H.; Lenigk, R.; Grodzinski, P. *Lab on a Chip* **2002**, *2*, 179−187.
(8) Lee, T. M.-H.; Carles, M. C.; Hsing, I.-M. *Lab Chip* **2003**, *3*, 100−105.
(9) Schneegass, I.; Bräutigam, R.; Köhler, M. *Lab Chip* **2001**, *1*, 42−49.
(10) Fukuba, T.; Yamamoto, T.; Naganuma, T.; Fujii, T. *Chem. Eng. J.* **2004** *101*, 151−156.

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amplified from *E. coli* genomic DNA and directly from untreated cells. For temperature control of their chip, six heaters made of ITO were placed on a glass substrate to act as three uniform temperature zones. Lee et al.[11] have recently reported a bulk-micromachined PCR chip. They validated that the proposed chip amplified the DNA related to the tumor suppressor gene BRCA 1 (127 bp at 11th exon) after 30 thermal cycles in a 200-nL-volume chamber. Although most of the recent assays are accurate and sensitive, they involve the definition of very stringent limits. The PCR products are usually separated by gel electrophoresis, and the band intensities are quantified by video imaging and densitometry. Additionally, Lagally et al.[12,13] have shown that microfluidic systems were capable of multiplexed PCR reactions and robust on-chip detection.

Microchamber arrays etched on silicon or glass were also one of the most reported miniaturized devices for multiple simultaneous DNA amplification.[14−16] The minimum reported size for a microchamber for PCR was demonstrated by Nagai et al.[15] in connection with PCR. Thus, the volume of a microchamber for a successful PCR amplification was reduced down to 86 pL. We have recently fabricated a highly integrated silicon microchamber array device and a precisely addressable DNA amplification system.[16] Loop-mediated isothermal amplification (LAMP)[17] was employed for the detection of target DNA in our previous report. Hepatitis B virus gene could be amplified in less than 1 h with high efficiency by using LAMP in connection with SYBR Green chemistry on our microchamber array. However, multiple-target DNA detection could not be performed; thus, our goal in this report was to achieve simultaneous detection of several numbers of target DNA.

Such a huge development for PCR chips was even accelerated by combining PCR with computerized laser technology, so that now the real-time monitoring of fluorescent signals for every PCR cycle took the place of the time-consuming postamplification steps. Namely, real-time PCR is the latest application of Mullis' "PCR" brainchild. It couples the two observations made in the mid-1990s that Taq DNA polymerase possessed 5′-nuclease activity[18] and fluorescent DNA binding dyes provided real-time monitoring.[19] The TaqMan system used in this report is based on the 5′−3′ exonuclease activity of the Taq DNA polymerase, which cleaves fluorescent dye-labeled probes during PCR; the intensity of fluorescence is then measured by a microarray scanner. The TaqMan probe is located between the two PCR primers and has a melting temperature 10 °C higher than that of the primers. The binding of the TaqMan probe prior to the primers is crucial because PCR products would then be formed without the generation of fluorescence intensity and thus could not be detected. A reporter dye, such as 6-carboxyfluorescein (FAM), is attached to the TaqMan probe. The emission spectrum of FAM is quenched due to the spatial proximity of a second fluorescent dye, 6-carboxytetramethylrhodamine (TAMRA). The degradation of the TaqMan probe by the Taq DNA polymerase releases the reporter dye from the quenching activity of TAMRA. Thus, the fluorescent signal increases with an increase in the cleavage of the probe, which is proportional to the amount of PCR product formed in the microchamber. The TaqMan principle is implemented in an Applied Biosystems (ABI) Prism Sequence Detection device (Foster City, CA). The ABI Prism 7700 is a laser-coupled spectrophotometer, which monitors the position of the 96-well microtiter plate. Most of the data are stored in a true real-time determination, and at the end of 40 cycles, all the data for quantitative analysis are stored in a computer file. In our chip, the quantitative analysis was also performed similarly to ABI Prism 7700 by using the microarray scanner data after the TaqMan PCR amplification in nanoliter volumes.

TaqMan PCR is the method of choice for our microarray-based chip because it uses internal probes for the quantification of PCR products and does not require different thermal cycling protocols. TaqMan also requires only a two-step procedure. Thus, the dispensing procedure and the preparation of the chip for amplification are greatly simplified and require fewer steps than it does for an ordinary PCR system. Additionally, TaqMan PCR provides trace DNA quantification with the robustness of a kinetic PCR and the advantages of the liquid hybridization assay but lacks the time-consuming postamplification steps. The hybridization step is carried out during amplification with no postamplification handling and thus reduces the overall manual handling and the risk of contamination and carryover. The most significant problem in the diagnostic application of PCR is the production of false-positive results. They are caused by the contamination of nucleic acids, particularly from previously amplified material (carryover). Any contaminant, even the smallest fragment carried over from a strongly positive sample (contamination), may be multiplied and produce a false-positive result. In the TaqMan system of our chip, the problem of carryover and contamination was significantly reduced by using the nanoliter-dispensing system.

In this report, the feasibility of our microchamber array was further improved by using TaqMan PCR. To the best of our knowledge, three different DNA sequences were amplified from three different DNA templates and detected in the same microchamber array simultaneously for the first time. In addition, the quantification of initial DNA concentration present in a microchamber was achieved from 0 to 12 copies per chamber, not only by monitoring the real-time fluorescence intensity but also by observing the end point fluorescence signal. Therefore, this system proves to be a promising device for the low-cost, high-throughput DNA amplification and detection for point-of-care clinical diagnosis, which can also be handled by nonspecialist users.

EXPERIMENTAL SECTION

Chip Fabrication. The silicon microchamber array chip was fabricated using protocols described in depth elsewhere.[16] Briefly, the dimensions of each microchamber were about 650 × 650 × 200 μm, the pitch of each microchamber was ∼900 μm and could accommodate ∼50 nL of sample volume. The chip had 1248

(11) Lee, D.-S.; Park, S. H.; Yang, H.; Chung, K.-H.; Yoon, T. H.; Kim, S.-J.; Kim, K.; Kim, Y. T. *Lab Chip* **2004**, *4*, 401−407.
(12) Lagally, E. T.; Emrich, C. A.; Mathies, R. *Lab Chip* **2001**, *1*, 102−107
(13) Lagally, E. T.; Medintz, I.; Mathies, R. A. *Anal. Chem.* **2001**, *73*, 565−570.
(14) Nagai, H.; Murakami, Y.; Yokoyama, K.; Tamiya, E. *Biosens. Bioelectron.* **2001**, *16*, 1015−1019.
(15) Nagai, H.; Murakami, Y.; Morita, Y.; Yokoyama, K.; Tamiya, E. *Anal. Chem.* **2001**, *73*, 1043−1047.
(16) Matsubara, Y.; Kobayashi, M.; Morita, Y.; Tamiya, E. *Arch. Histol. Cytol.* **2002**, *65*, 481−488.
(17) Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T. *Nucleic Acids Res.* **2000**, *28*, E63.
(18) Eom, S. H.; Wang, J.; Steitz, T. A. *Nature* **1996**, *382*, 278−281.
(19) Livak, K. J.; Flood, S. J.; Marmaro, J.; Giusti, W.; Deetz, K. *PCR Methods Appl.* **1995**, *4*, 357−362.

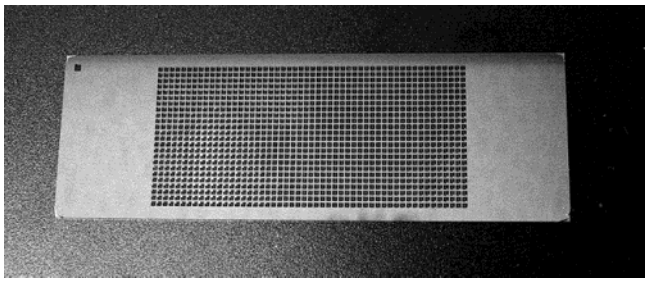


Figure 1. Photographic image of the microchamber array chip. The 1248 microchambers are integrated on the 1 in. × 3 in. of a silicon chip.

chambers in total (Figure 1). The chip feature was 1 in. × 3 in. To achieve precise introduction of sample mixture into the microchamber, only the inner wall surfaces of the microchamber were prepared as hydrophilic by leaving an oxidized layer on them with photolithographic techniques, as recently described by Felbel et al.[20]

Reagents and Sample Preparation. A 295-bp segment of the human β-actin,[21] a 137-bp segment of the human sex-determining region Y (SRY),[22] and a 74-bp segment of the human Rhesus D (RhD)[23] were amplified, respectively. The primers and probe sequence specific to the β-actin gene were as follows: forward, 5′-TCA CCC ACA CTG TGC CCA TCT ACG A-3′; reverse, 5′-CAG CGG AAC CGC TCA TTG CCA ATG G-3; probe, 5′-ATG CCC *T*CC CCC ATG CCA TCC TGC GT-3′ (Applied Biosystems). This probe was labeled at the 5′ end with the fluorescent reporter FAM and at the italic base (T, thymine) with the quencher TAMRA. The following primers and probe were used to amplify, and detect the SRY gene: forward, 5′-TGG CGA TTA AGT CAA ATT CGC-3′; reverse, 5′-CCC CCT AGT ACC CTG ACA ATG TAT T-3′; probe, 5′-AGC AGT AGA GCA GTC AGG GAG GCA GA-3′. The following were used to amplify and detect the RhD gene: forward, 5′-CCT CTC ACT GTT GCC TGC ATT-3′; reverse, 5′-AGT GCC TGC GCG AAC ATT-3′; probe, 5′-TAC GTG AGA AAC GCT CAT GAC AGC AAA GTC T-3′ (FASMAC Co., Kanagawa, Japan). These two probes were labeled at the 5′ end with FAM and at the 3′ end with TAMRA.

The reaction mixture consisted of 1× GeneAmp PCR buffer II, 0.2 mM each dNTP, 4.5 mM $MgCl_2$, and 0.1 unit/μL AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.5% (w/w) poly(vinylpyrrolidone) (Sigma-Aldrich, Tokyo, Japan), and a desired concentration of the template DNA. As the template DNA, several concentrations of human male and female genomic DNA except as stated otherwise (Promega, Tokyo, Japan) and also human Rh (−) female DNA, which was kindly donated by Dr. Takabayashi in Kanazawa Medical University, were used by dilution with deionized water. Additionally, three kinds of primer and probe mixtures were also prepared. The primer and probe mixtures consisted of 600 nM forward and reverse primers and also 400 nM labeled probe, respectively.

Sample Loading with a Nanoliter Dispenser. First, the microchamber array chip was soaked in 1% (w/v) bovine serum albumin (BSA) solution overnight, then rinsed with deionized water, and dried in order to prevent nonspecific adsorption by coating the chamber wall. The chip was placed onto the dispensing stage of a nanoliter dispenser from Cartesian Technologies. The precise dispensing of nanoliter volumes of solutions exactly at previously determined locations had become very simple by using their technology. The volume of dispensed solution in a single microchamber was 40 nL. The mineral oil (Sigma, Tokyo, Japan) as a cover lid was coated onto the template DNA-modified chip, and 40 nL of PCR mixture, which included β-actin-specific primers and probe, was dispensed into all of the microchambers through the oil layer. After preparing this setup, the chip was placed onto a conventional thermal cycling system to achieve PCR reaction. Thermal cycling was initiated with a 94 °C and held for 10 min, followed by 40 cycles of 94 °C for 10 s and 60 °C for 60 s. After the end of PCR amplification, the amplified DNA was observed using a charge-coupled device (CCD) camera (Hamamatsu Photonics, Tokyo, Japan), which was mounted on a fluorescence microscope (Leica, Heidelburg, Germany).

Multiple DNA Amplification and Detection. Three kinds of specific primers and dual-labeled probes related to β-actin, SRY, and RhD were introduced into a certain area (12 × 4) of the microchambers one by one by using the nanoliter dispenser and dried at room temperature (Scheme 1a). After preparing the oil lid (Scheme 1b), three kinds of PCR mixtures, which included template DNA (human male DNA, human female Rh (−) DNA, or negative control DNA), were dispensed into another specific area (4 × 12) of the microchamber through the oil layer (Scheme 1c). DNA amplification was performed with the same setup as described above. The amplified DNA was observed using not only a CCD camera with fluorescence microscope but also a fluorescence image scanner and its software purchased from Hitachi Co. (Tokyo, Japan) which were employed in order to evaluate and analyze fluorescence intensity of each chamber. SmartCycler real-time PCR system was obtained from Cepheid Technologies. The TaqMan PCR protocol for the amplification of SRY, RhD, and β-actin genes was performed as described above.

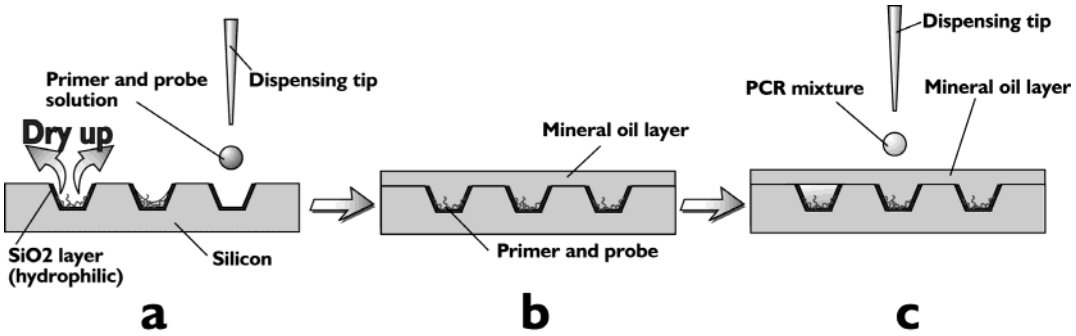
Quantification of Initial DNA Concentration. While we were splitting the primers and probe solution, which were specific for the SRY sequence, into smaller aliquots, we have used a DNA analyzer system BioSpec from Shimadzu Inc. (Tokyo, Japan) in order to quantify our DNA samples. Thus, all the concentrations of the aliquots were checked and confirmed before dispensing into six different areas of the microchamber array. Each area consisted of 60 microchambers; 40 nL of the solution was introduced precisely into each microchamber. The PCR mixtures including different concentrations of target DNA, 0, 0.4, 2.0, 4.0, 8.0, and 12 copies/40 nL, were prepared, and then the mixtures were also introduced into 60 microchambers, after coating the chip surface with the mineral oil layer. The detection of the amplified DNA was performed as described above.

RESULTS AND DISCUSSION

Target DNA sequence was amplified specifically in a nanoliter-volume microchamber, and the microchambers, in which fluorescence signal was released, were counted in consequence to TaqMan PCR. The inner walls of the microchamber were rendered

(20) Felbel, J.; Bieber, I.; Pipper, J.; Köhler, J. M. *Chem. Eng. J.* **2004**, *101*, 333−338.
(21) Kalinina, O.; Lebedeva, I.; Brown, J.; Silver, J. *Nucleic Acids Res.* **1997**, *25*, 1999−2004.
(22) Skinner, J.; Luettich, K.; Ring, M.; O'Leary, J. J.; Turner, M. J. *Obstet. Gynecol.* **2001**, *97*, 460−463.
(23) Turner, M. J.; Martin, C. M.; O'Leary, J. J. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2003**, *108*, 29−32.

Scheme 1. Illustration of Preparation Steps for On-Chip DNA Amplification[a]



[a] (a) Different types of primers were dispensed into the microchamber and then dried. (b) The mineral oil was coated on the chip after the primer solution was dried. (c) PCR mixture of 40 nL with no primers was dispensed in each chamber through the oil layer. The solution sunk to the bottom and then spread to the microchamber walls.

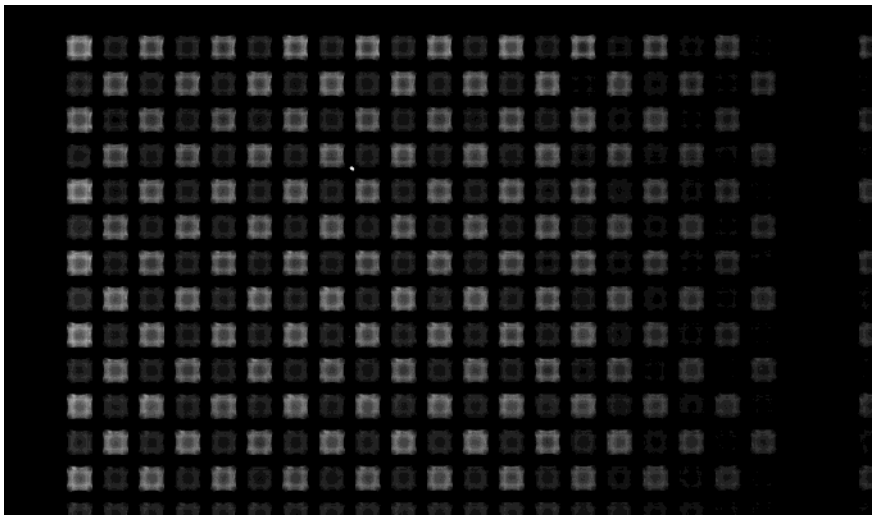


Figure 2. Photographic image of fluorescence via TaqMan PCR using alternate dispensings of microchambers containing template and those without template. Only the microchambers, into which template DNA was dispensed, showed the release of positive fluorescence signal.

hydrophilic with the formation of an oxidized layer on their surfaces. After coating the microchamber array with mineral oil, the remaining hydrophobic surface of the microchamber prevented the spread of the aqueous solution to the outside of the microchamber. After dispension of the aqueous sample solution, it first formed a droplet, which in time was replaced with the oil in the microchamber, and settled inside the microchamber with the convection of the oil as illustrated in Scheme 1. The thickness of the oil layer had a significant effect on the protection of nanoliter-scale solutions from evaporation. The oil layer was adjusted by controlling the volume of the oil drop. As the thickness of the oil layer increased, the dispensing of the sample solution became more erroneous (data not shown). The optimum thickness of the oil layer for introduction of the sample mixture was chosen to be $\sim$200 μm.

There were several inhibition factors, such as nonspecific adhesion of biomaterials, variety in the distribution efficiency of the sample dispensing, and the errors caused by PCR itself. Surface treatment of the microchamber was also an important factor affecting the efficiency of the PCR reaction. Several kinds of surface treatment methods were reported by Shoffner et al.[24] to be useful for avoiding the adsorption of biomaterials on the silicon surface. Erill et al.[25] reported a systematic analysis of material-related inhibition and adsorption phenomena in glass−silicon PCR chips. Their results suggested that the previously reported inhibition of PCR by silicon-related materials was caused mainly by the adsorption of Taq polymerase at the walls of the chip due to increased surface-to-volume ratios; thus, a straight chemical action of silicon-related materials on the PCR mixture was negligible. In contrast to Taq polymerase, DNA was not found to be adsorbed in significant amounts. The net effect of polymerase adsorption could be prevented by the addition of a titrated amount of a competing protein, BSA, and the ensuing reactions could be kinetically optimized to yield efficient PCR amplifications. In our system, we combined these advantageous points of previous reports. The surface of the microchamber walls was first modified by an oxidized layer[20,24] and then coated with BSA.[25] Only a very low fluorescence signal could be observed, when no BSA coating was employed. Thus, it was found necessary to coat the oxidized walls of the microchambers with BSA in good agreement with the findings of Erill et al.[25] To quantify DNA concentration, a certain number of microchambers were used as one region for only one concentration. Thus, 60 microchambers were used to determine the variations in the fluorescence signal of the dis-

(24) Shoffner, M. A.; Cheng, J.; Hvichia, G. E.; Kricka, L. J.; Wilding, P. *Nucleic Acids Res.* **1996**, *24*, 375−379.

(25) Erill, I.; Campoy, S.; Erill, N.; Barbé, J.; Aguiló, J. *Sens. Actuators, B* **2003**, *96*, 685−692.

pensed one specific concentration of target DNA in our further studies.

The cross-contamination between chambers was tested by using alternate dispensings of wells containing template and those without template as shown in Figure 2. A high concentration of the template DNA (160 pg/chamber) was introduced as alternate dispensings into the microchambers. Fluorescence signals were obtained only from the chambers, into which template DNA was introduced. No fluorescence was gained from the remaining chambers, into which no template DNA was dispensed (Figure 2). Thus, it was concluded that the selective distribution of the template DNA into the microchambers was achieved in our system.

A comparison of amplification efficiency was made between two different DNA modification methods. The template DNA was dispensed onto the oil-coated chip before and after dispension of the PCR mixture including the primers and the labeled probe. The fluorescence intensity after the TaqMan PCR was observed to be almost the same in both modification methods. This result suggested that the amplification efficiency was not affected by the sequence of the reagent dispension.

Figure 3A shows the fluorescence image of the chip after DNA amplification of three different target DNA sequences from three different DNA templates. Visual comparison of the positive fluorescence intensity signals with the negative ones greatly simplified the procedure to distinguish which chamber contained the target DNA. If the target DNA sequence was present in the dispensed sample, a high fluorescence signal was easily obtained in consequence to TaqMan PCR. Additionally, the background fluorescence intensity of the β-actin PCR system was found to be much lower than the one of other two probes by using both our chip and the SmartCycler real-time PCR system (Figure 3B). SmartCycler real-time PCR system results were in good agreement with the results of our chip. The difference in the background fluorescence intensity was caused by the bp distance between FAM and TAMRA dyes of the TaqMan probes. In the TaqMan probe for β-actin gene, FAM was only 6 bp away from TAMRA, but FAM and TAMRA were 26 and 31 bp away from each other in the probes for SRY and RhD genes, respectively. Such a short distance of 6 bp between the dyes caused the rapid quenching of the signal, and thus, the β-actin system could release much lower fluorescence signals after amplification in comparison with the other systems. Although the PCR systems in this experiment had such different background fluorescence intensities, accurate detection of TaqMan amplification for all systems was achieved by using our chip. Since Rh(−) human female genomic DNA did not contain SRY and RhD genes, almost none of the microchambers showed a fluorescence signal. Both human male and female genomic DNA contained the β-actin gene; thus, a fluorescence signal could be observed successfully in all microchambers of the related area on the chip. The fluorescence intensity of the microchambers was also scanned and evaluated using a DNA microarray scanner and its analysis system. Figure 3C shows the fluorescence intensity values obtained from 16 chambers. The remarkable difference between the fluorescence intensities of the positive and negative controls was observed clearly for all three DNA templates. This result indicated that our system was capable of detecting different kinds of target DNA sequences from different

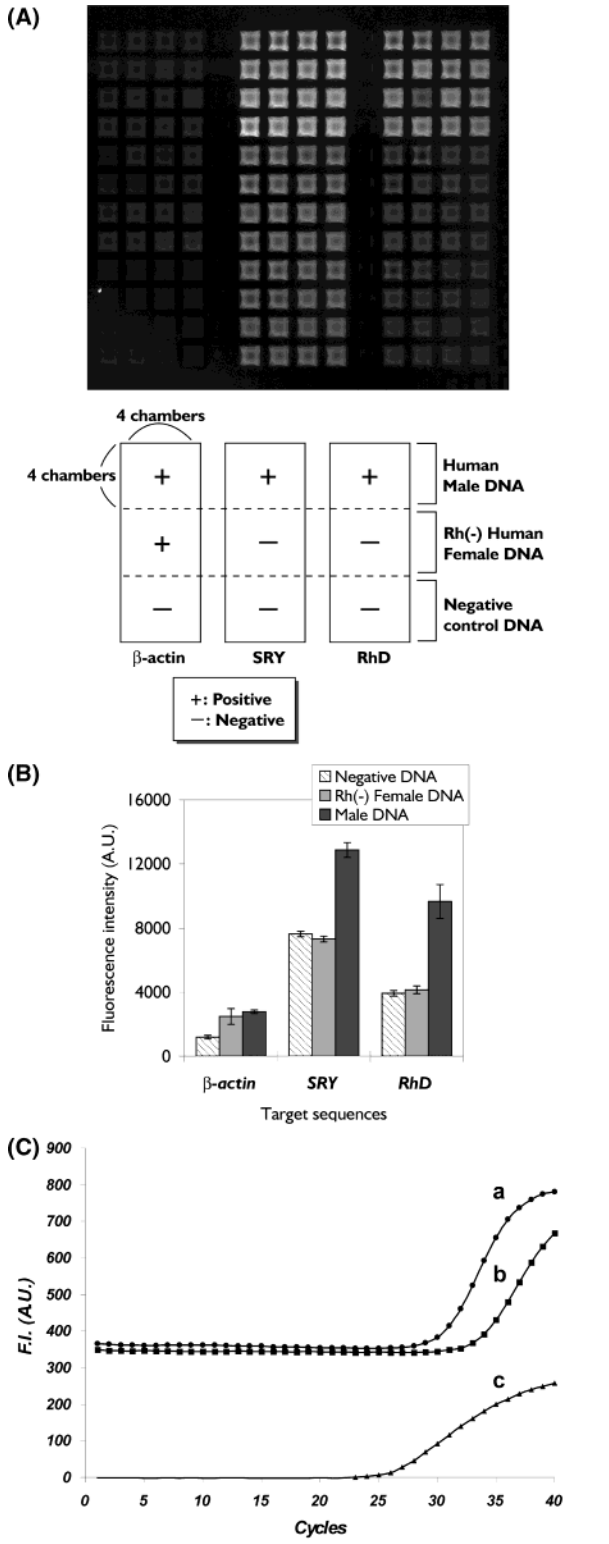


Figure 3. Multiple PCR analysis of β-actin, SRY, and RhD genes using three different kinds of template DNA. (A) Photograph of the microchamber array in consequence to multiple TaqMan PCR. The scheme shows an illustration for the outcome of the fluorescence responses from the specific areas of the microchambers, where positive or negative samples from different sources were dispensed. (B) Average fluorescence intensity values obtained from 16 microchambers. The remarkable difference between the fluorescence intensities of the positive and negative controls is clearly presented for three target DNA sequences. (C) Real-time fluorescence data for (a) SRY, (b) RhD, and (c) β-actin amplification obtained from SmartCycler system.

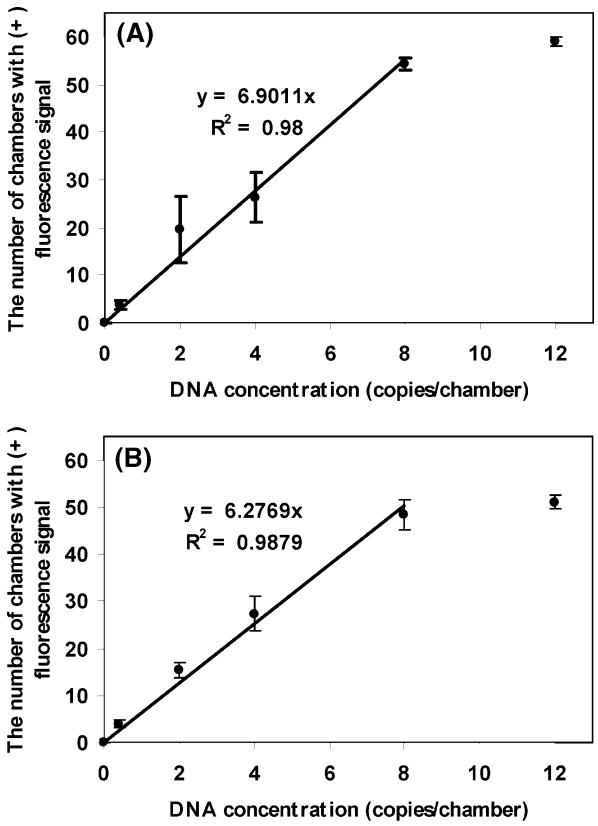


Figure 4. Number of microchambers with positive fluorescence signal versus the number of initial target copies in one chamber for quantitative analysis. Data were obtained from three consequtive experiments of microchamber array PCR. The large R^2 value for (A) SRY ($R^2 = 0.98$) and (B) RhD ($R^2 = 0.988$) indicates that there is a linear correlation from 0 to 8 copies/microchamber; a plateau region is obtained above 8 copies/microchamber.

DNA sources simultaneously. Since TaqMan PCR required the same thermal cycling protocol for the amplification of many kinds of target sequences, it was found to be the most suitable detection technique for microchamber array PCR systems in this report. For example, the detection of genetic diseases such as Down's syndrome,[26] 22q11.2 deletion syndrome,[27] and β-thalassaemia[28] using TaqMan PCR has already been reported. The simultaneous detection of these clinically important diseases can also be performed by using our microchamber array-based PCR chip.

Figure 4 plots the number of chambers with positive fluorescence signal versus input template DNA copy number for the amplification of SRY (A) and RhD (B) sequences. As target DNA increased from 0 to 12 copies/chamber, the number of microchambers with a positive fluorescence signal also increased. It was possible to fit the data from 0 to 8 copies/chamber into a straight line with the regression coefficients of 0.98 and 0.9879 for SRY and RhD sequences, respectively. The system reached a saturation plateau after DNA concentration of 8 copies/chamber, indicating that trace amount of target DNA was satisfactory for the detection process. PCR amplification in almost the whole block of the chip was achieved by using eight copies of the target DNA. Such a behavior indicated the high detection capacity of our system, so that even a trace amount of DNA copies would be satisfactory for a precise quantification. The copy numbers above eight that would be amplified without any problems, however, could not be quantified. Even 0.4 copies of the target DNA was enough to give a readable signal, which was determined as our limit of detection. The average fluorescence intensity value of 1000 AU was determined as the threshold. When 0.4 copies were dispensed, only 2 microchambers reached within the 1000−1200 AU level. One would have expected to get 4 out of 10 microchambers to be positive and 6 to be negative, if we had an average of 0.4 copies/microchamber, and every copy was intact. The polymerase concentration, annealing temperature, $MgCl_2$ concentration, and the specific primers and TaqMan probes should have been kept under the optimum conditions in 40-nL volumes in the microchamber, which was a tedious task. The instrumental limitations also added to the inefficiency of PCR, when such a small volume containing such a trace amount of analyte was dispensed on a microchamber array. Thus, the combined negative effects of biochemical (resulting from the TaqMan PCR itself) and instrumental limitations caused the appearance of only 2 microchambers out of 60 with positive signals after TaqMan PCR on our system.

Our method requires only counting the microchambers, which show a positive fluorescence signal, in consequence to PCR amplification. No special equipment for the detection of real-time fluorescence intensity is required for determination of DNA copy numbers in this report. Only a simple fluorescence microscope, or a transilluminator used for gel electrophoresis, can be employed for accurate observation of the fluorescence released microchambers with positive signals.

CONCLUSIONS

The microchamber array chip presented in this report could be used to amplify multiple DNA targets in combination with a nanoliter dispenser. Theoretically, the chip could be used to amplify and detect ~1200 target DNA simultaneously. The size and the total number of the microchambers are determined by the dispensing system. The minimum solution volume, which could be dispensed with reliability, was optimized as 40 nL for our experiments. If the dispensing instrument could be improved to provide the dispension of a lesser volume of solution, the size of microchamber would become smaller, and the chip would become more integrated with a higher number of microchambers. Such further integration of our microarray PCR chips with a miniaturized thermal cycler unit is in progress in our laboratory. The microarray PCR chip reported here has a significant potential to be implemented for a wide range of applications. Overall, this system is a promising candidate for mass microfabrication due to its low-cost and high-throughput detection ability.

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(26) Zimmermann, B.; Holzgreve, W.; Wenzel, F.; Hahn, S. *Clin. Chem.* **2002**, *48*, 362−363.
(27) Kariyazono, H.; Ohno, T.; Ihara, K.; Igarashi, H.; Joh-o, K.; Ishikawa, S.; Hara, T. *Mol. Cell. Probes* **2001**, *15*, 71−73.
(28) Chiu, R. W.; Lau, T. K.; Leung, T. N.; Chow, K. C.; Chui, D. H.; Lo, Y. M. *Lancet* **2002**, *360*, 998−1000.