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Analysis of Specific Gene by Integration of Isothermal Amplification and Electrophoresis on Poly(methyl methacrylate) Microchips

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We have successfully achieved the integration of isothermal amplification and the subsequent analysis of specific gene fragments on poly(methyl methacrylate) microchips. In our experiments, loop-mediated isothermal amplification, which can offer higher specificity and efficiency than PCR, has been performed at a constant temperature (65 °C). After amplification, products could be either examined by the integrated microchip-based electrophoresis or directly observed by naked eye with SYBR Green I added into the reaction solution. By such an integrated microsystem, the amplification and the subsequent analysis of prostate-specific antigen gene with template concentration at 23 fg/μL could be finished within 15 min, which demonstrates its advantages of high specificity, good reproducibility, and fast speed in gene detection.

Sequencing of the human genome has been almost completed, and the human genome project quickly moves on to the postgenome-sequencing era.[1−4] During this period, further development of analytical technology is highly required for high-throughput screening of disease-causing genes and high-speed analysis of genetic polymorphism on an individual genome. Accordingly, the development of novel microchip-based methods for ultrafast DNA analysis has been challenged, especially for the amplification and analysis of genetic locus on the genome.[5−8]

Since its initial study from the middle of 1980s, PCR has been playing an important role in modern biology research.[9] During the amplification, thermal cycling with temperature ranging from 50 to 100 °C is indispensable. Therefore, most reported PCR chips are fabricated by either glass or silicon.[10−13] However, polymer microchips, which are expected to be very promising in the future,[14] can hardly endure the high temperature during PCR. Accordingly, amplification at a relatively low temperature should be especially developed for them.

Up until now, several isothermal amplification methods under mild conditions have been proposed, including nucleic acid sequence-based amplification,[15] self-sustained sequence replication,[16] and strand displacement amplification.[17,18] Each of them has its own innovation to reinitiate new rounds of DNA synthesis, but there are still drawbacks to overcome.[19,20] They require either a precision instrument for amplification or an elaborate method for detecting the amplified products due to poor specificity of target sequence selection. Loop-mediated isothermal amplification (LAMP) is another newly invented method that can amplify a few copies of DNA to 10^9 within 1 h under isothermal condi-

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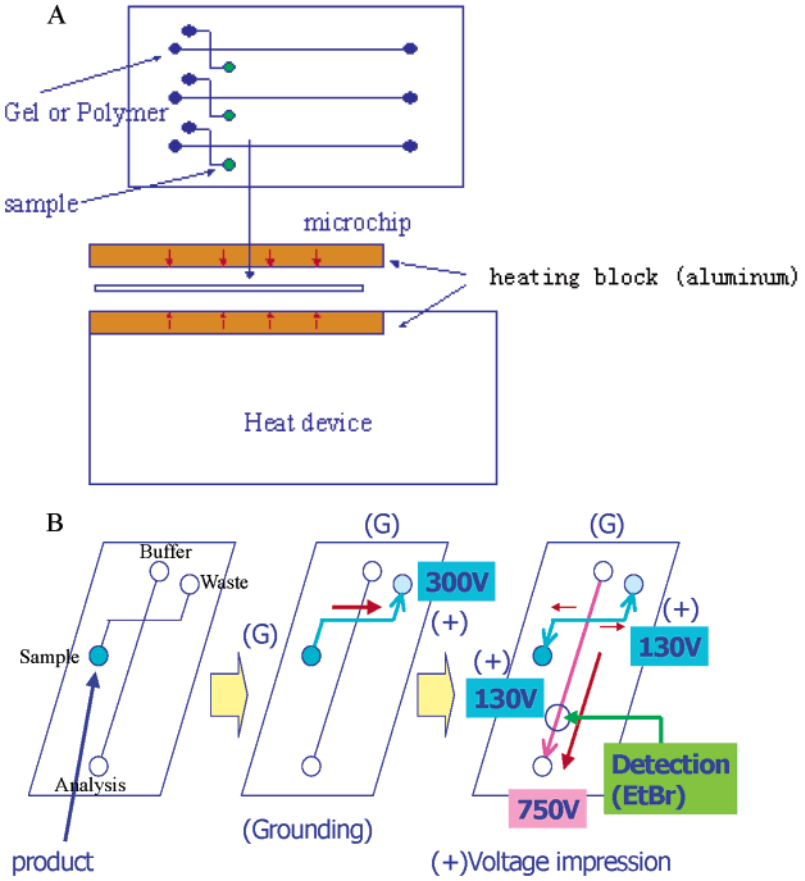

Figure 1. On-chip integration of LAMP and electrophoresis-based analysis of products. (A) Schematic diagram of microsystem; (B) electrophoresis-based detection of amplification products.

tions (50−65 °C) with great specificity.[21−24] Therefore, LAMP might be quite suitable for genetic amplification on polymer microchips.

In this paper, to the best of our knowledge, for the first time, we have successfully integrated LAMP and the subsequent detection of amplification products on poly(methyl methacrylate) (PMMA) microchips. Through its application in the analysis of some specific DNA fragments, such an integrated microsystem has shown the advantages of rapidity, high sensitivity, high efficiency, and high specificity.

EXPERIMENTAL SECTION

Apparatus. LAMP on a microchip was performed on a Block Incubator (Astec, Fukuoka, Japan). Analysis of amplification products by microchip electrophoresis was carried out on a Hitachi SV 1100 instrument (Hitachi Electronics Engineering Co., Tokyo, Japan). A commercial PMMA microchip for LAMP and electrophoresis (Hitachi, Tokyo, Japan) had a simple cross channel of 100-μm width and 30-μm depth. The effective separation length was 30 mm, and the distances from the channel crossing point to the sample, waste, buffer, and analysis wells were 5.25, 5.25, 5.75, and 37.5 mm, respectively.

Reagents. All reagents for LAMP were kindly donated by Eiken Chemical Co. Ltd. (Tochigi, Japan). SYBR Green I was bought from Molecular Probes, Inc. (Eugene, OR). DNase and RNase-free water (ICN Biomedicals, Inc.) were used throughout our experiments.

Reaction Mixture for LAMP. A 569-bp fragment of prostate-specific antigen (PSA) cDNA was amplified by PCR using primers of 5′-CGGGATCCAGCTGTGTCACCATGTGGGT and 5′-CGGGATCCTGCGCACACACGTCATTGGAA. Then the products were digested with *Bam*HI, and the digested PCR products were inserted in *Bam*HI-digested pBluescript before being used as the template. λ-DNA (Takara Shuzo) was also used as another template.

The amplified regions for PSA and λ-DNA were respectively 136 and 132 bp. The primers used for PSA cDNA amplification were PSA BIP (5′-TGTTCCTGATGCAGTGGGCAGCTTTAGTCTGCGGCGGTGTTCTG-3′), PSA FIP (5′-TGCTGGGTCGGCACAGCCTGAAGCTGACCTGAAATACCTGGCC TG-3′), PSA B3 (5′-TGCTTGTGGCCTCTCGTG-3′), PSA F3 (5′-GGGTGTGGGAAGCTGTG-3′). Those for λ-DNA amplification were λ FIP (5′-AGGCCAAGCTGCTTGCGGTAGCCGGACGCTACCAGCTTCT-3′), λ F3 (5′-AAAACTCAAATCAACAGGCG-3′), λ BIP (5′-CAGGACGCT GTGGCATTGCAGATCATAGGTAAAGCGCCACGC-3′), and λ B3 (5′-GACGGATATCACCACGATCA-3′).

On-chip LAMP was carried out in the sample well on the microchip with a total reaction volume of 10 μL, containing 8 units of Bst DNA polymerase (New England Biolabs), 0.8 μM each FIP

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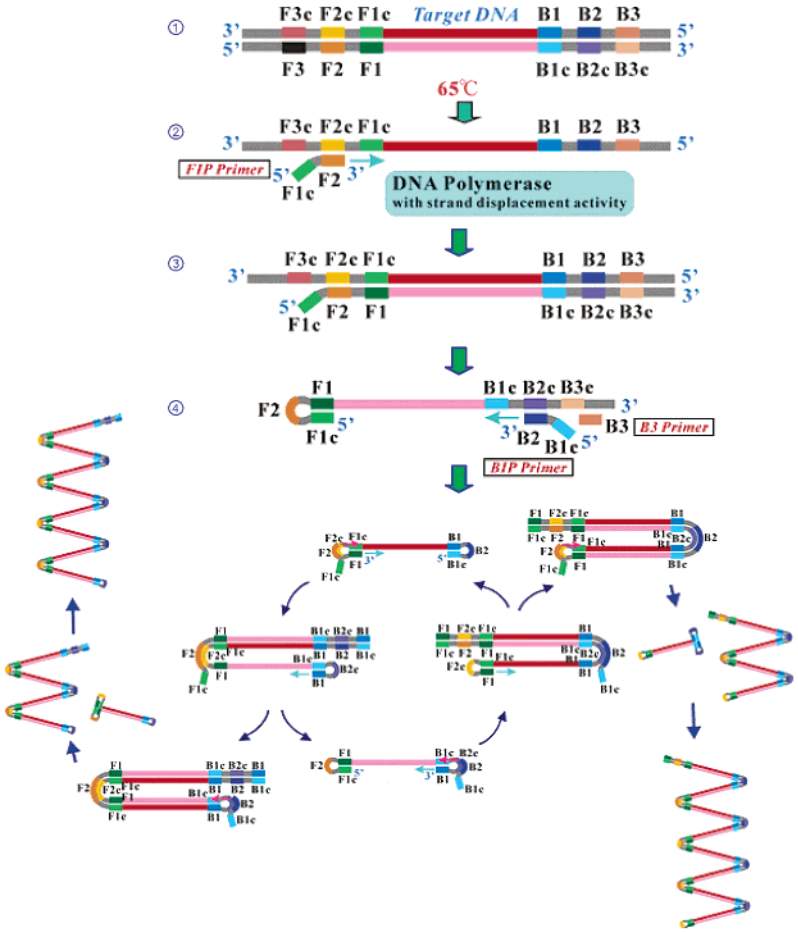

Figure 2. Principle of LAMP.

and BIP, 0.2 μM each F3 and B3, 400 μM each dNTP, 1 M betaine (Sigma), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 0.1% Triton X-100, and various amounts of double-stranded DNA.

Procedures. Polymer solution from a Hitachi SV1100 kit was introduced into the microchip channels via a syringe. Then the amplification solution was added into the sample well. The remaining three reservoirs were filled with the same polymer solution as that in the channel. Before the whole microchip was incubated at 65 °C (Figure 1.A), all wells were covered with Thermowell seals (Costar). After amplification, the products were examined by either microchip electrophoresis-based separation with the procedure shown in Figure 1B or direct observation by eye on condition that fluorescent dye was added into the reaction solution.

RESULTS AND DISCUSSION

Principle of LAMP. The LAMP method relies on autocycling strand displacement DNA synthesis that is performed by a DNA polymerase with high strand displacement activity and a set of two specially designed inner and two outer primers.[21] In the initial steps of the LAMP reaction, all four primers are used, but later during the cycling reaction, only the inner primers are used for strand displacement DNA synthesis. The mechanism and expected reaction steps of LAMP are illustrated in Figure 2. An inner primer containing sequences of the sense and antisense strands of the target DNA initiates LAMP. The following strand displacement DNA synthesis primed by an outer primer releases a single-stranded DNA. This serves as a template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem−loop DNA structure. In subsequent LAMP cycling, one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem−loop DNA and a new stem−loop DNA with a stem twice as long. The cycling reaction continues with accumulation of 10^9 copies of target in less than 1 h. The final products are stem−loop DNAs with several inverted repeats of target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. However, if there is no target DNA template in the sample, the amplification could not be performed, and thus, only primer or primer dimer peaks could be detected after the reaction. Because LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterward, it is expected to amplify the target sequence with high selectivity.

Integration of LAMP and Electrophoresis Analysis on Microchip. With the consideration of the optimal amplification temperature of LAMP and the glass transition temperature of PMMA, isothermal amplification and the subsequent electrophoresis-based analysis of 23 fg/μL PSA cDNA are integrated on a PMMA microchip at a constant temperature of 65 °C. In our experiments, PMMA chip is compatible with all reagents in a

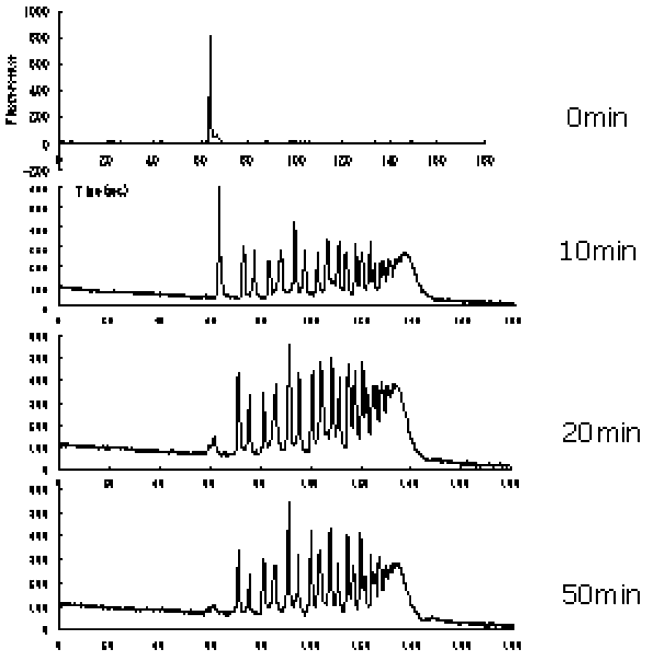

Figure 3. Analysis of amplification products by the integrated microsystem.

LAMP assay. From Figure 3, it could be seen that, at the beginning, only one peak is detected, which is the mixture of four primers, primer dimers, or both. After 10 min, this peak decreases obviously, and the amplified DNA fragments with various sizes could be seen, which shows the high speed of on-chip LAMP. In addition, the examination of the amplification products by microchip electrophoresis could be finished in 150 s; i.e., the total time spent on the amplification and detection of the amplified products of PSA cDNA is just within 15 min, which further demonstrates the rapidity and high efficiency of such an integrated microsystem in gene analysis. Furthermore, with the amplification time increased to 20 min, more products could be detected. However, after 50 min of reaction, no increase of products could be seen, which means that the reaction might be finished by the depletion of primers.

To examine the reproducibility of our developed integrated microsystem, the amplification and the subsequent electrophoresis-based analysis of amplification products have been repeated four times on PMMA microchips. Figure 4 shows that the fluorescence intensity and the size of amplified PSA gene fragments are quite similar to each other, which indicates that such an integration is of good reproducibility.

Besides the study of the PSA gene, such an integrated microsystem has also been applied to the amplification of λ-DNA. Although the target products of PSA gene and λ-DNA only have a 4 bp difference in length (136 and 132 bp, respectively), the DNA profiles are quite different from each other (Figure 5), which proves the high specificity of LAMP. In addition, with the mixture of PSA gene and λ-DNA as templates, the amplified products are also not the same as those obtained by each respective reaction. Accordingly, based on the comparison of DNA profiles of known and unknown samples, we can examine whether the templates are same or not, which is quite useful in clinical diagnostics, especially for mutation analysis.

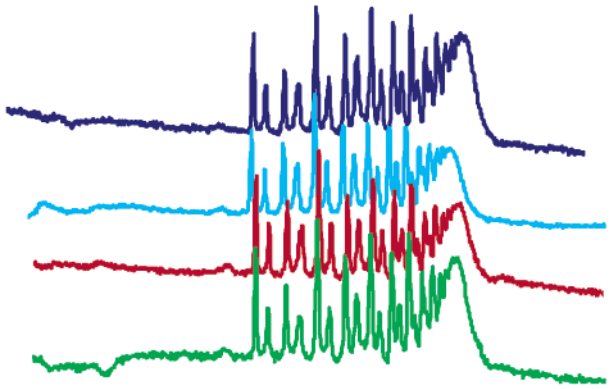

Figure 4. Reproducibility of LAMP products for PSA gene after 10-min amplification.

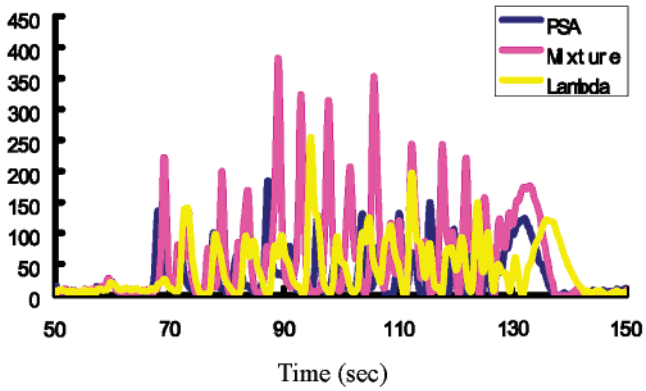

Figure 5. Detection of PSA, λ-DNA, and their mixture.

Direct Observation of On-Chip LAMP Products. Since the amplification efficiency of LAMP is 10−100 times as high as that of PCR,[21] the product concentration obtained by LAMP is markedly higher than that obtained by PCR. Accordingly, it is possible to check the existence of amplified DNA fragments by directly observing the color variance of fluorescence dye that has been added into the reaction solution, so that the detection of on-chip LAMP products could be greatly simplified.

In our experiments, it has been observed that the addition of some fluorescent dyes, especially at high concentration, into LAMP reaction solution might inhibit the amplification. Therefore, different dyes are individually added into the sample well after on-chip amplification. Through comparing the color change between sample and control solution, SYBR Green I is finally chosen as the detection fluorescent dye. Figure 6 shows the comparison results with 100× SYBR Green I added into the control solution and amplification products, respectively. Just with the naked eye, we can see that, in the control experiment, the solution is beige. However, with DNA present, the color changes to green. Accordingly, in this way, no additional complex detector is required.

From the above-mentioned result, it could be predicted that, with on-chip LAMP, product visualization by means of fluorescent dye is useful to judge the existence of target nucleic acids, especially for quick inspection and diagnosis in clinical diagnostics. In addition, such smart DNA check can also be applicable to develop portable gene analysis instruments for detecting target DNA of O-157 *Escherichia coli*, *Bacillus anthracis*, *Salmonella*, and other bacteria in the environment, food, animal, etc.

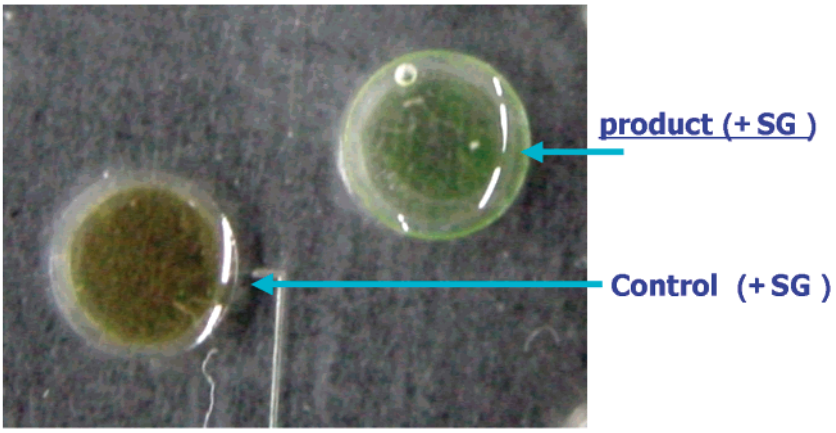

Figure 6. Observation of PSA gene by eye.

CONCLUSION

As an isothermal amplification method with high specificity, efficiency, and rapidity, LAMP has proven suitable to perform on polymer microchips. In addition, microchip-based electrophoresis can offer high sensitivity, efficiency, and speed to analyze amplification products. Accordingly, the integration of these two techniques is superior in the detection of specific nucleic acids. All these results demonstrate such an integrated microsystem is of great potential in clinical diagnostics, environment monitoring, and food quarantine. Furthermore, the on-chip amplification products by LAMP could also be observed directly by eye when suitable fluorescent dye was added after reaction, which could greatly simply the detection of genes.

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