Microchip-based one step DNA extraction and real-time PCR in one chamber for rapid pathogen identification

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Optimal detection of a pathogen present in biological samples depends on the ability to extract DNA molecules rapidly and efficiently. In this paper, we report a novel method for efficient DNA extraction and subsequent real-time detection in a single microchip by combining laser irradiation and magnetic beads. By using a 808 nm laser and carboxyl-terminated magnetic beads, we demonstrate that a single pulse of 40 seconds lysed pathogens including *E. coli* and Gram-positive bacterial cells as well as the hepatitis B virus mixed with human serum. We further demonstrate that the real-time pathogen detection was performed with pre-mixed PCR reagents in a real-time PCR machine using the same microchip, after laser irradiation in a hand-held device equipped with a small laser diode. These results suggest that the new sample preparation method is well suited to be integrated into lab-on-a-chip application of the pathogen detection system.

1. Introduction

In developing a pathogen detection system, efforts have been mainly focused on the detection of the pathogen genome very sensitively and accurately. In cases such as *Bacillus anthracis*, a Gram-positive bacterium that forms a rigid spore, or *Streptococcus mutans*, a Gram-positive non-spore-forming anaerobic bacterium with thick peptidoglycan cell walls, the lysis efficiency is the key to successful detection.[1] For the general usage of the pathogen detection system, it is important to disrupt any kind of pathogen and purify the target DNA rapidly prior to the detection using PCR (polymerase chain reaction). In particular, it is critical to reduce the processing time and the number of steps for the quick detection of pathogens. Pal *et al.* tested an integrated microfluidics device for influenza detection and subtyping, and insisted that lysis and purification were still off-chip considering the different types of sample sources.[2] Thus, the integration of sample pretreatment such as cell lysis and purification into microfluidic devices represents one of the most formidable hurdles yet to be solved in lab-on-a-chip (LOC) devices.

One of the cell lysis methods that have been tried on a microsystem to speed up the processing for DNA isolation is sonication. Sonication with an ultrasonic horn is an alternative physical method; lysis of anthrax spores was performed using a minisonicator system to extract DNA for the PCR amplification.[3] Subsequent work performed by Taylor *et al.*[4] used the same sonicator system with the addition of beads which were vigorously shaken, causing efficient cell disruptions. Although ultrasonic disruption for Gram-positive bacterial cells and spores has been efficient, this method has several disadvantages for cell lysis on LOC, including non-uniform energy distribution, auditory disturbance to human ears, the large ultrasonic transducer and high power consumption of ultrasonic operation. The chemical method employs harsh chemical substances to disrupt cell walls and requires additional treatments with chaotropic reagents to denature proteins. A disadvantage of chemical lysis methods is that these chemical reagents could interfere with the subsequent PCR reaction, which results in a reduced yield of DNA recovery. This is also labor-intensive and requires expensive consumables. A thermal method involves repeated freeze–thaw cycles but often fails to disrupt many structures inside the cells. Heating is an alternative method for disrupting cell walls or membranes. The problem of simple heating is that the denatured proteins can interact with the released DNA and thus interfere with the following DNA amplification.[5] A mechanical pressing instrument is not suitable for the LOC application because it is bulky and expensive.[6,7] The electrical method would be very useful if the electrodes are present within the LOC. However, the disadvantage of the electrical method is the need for a high electric field power (up to 2 kV cm^{-1}) to disrupt the cells.[8–10]

In this paper, we have developed a unique sample preparation method using Laser-Irradiated Magnetic Bead System (LIMBS) for efficient cell lysis and DNA isolation from various pathogens. We studied the basic mechanism of the cell disruption by LIMBS to optimize the conditions for the cell lysis and DNA release. It is noteworthy that the Gram-positive bacterial cells (*Streptococcus mutans*, *Staphylococcus epidermidis*) as well as Gram-negative bacterial cells (*Escherichia coli* BL21) were easily disrupted within 40 seconds in the microchip.

The use of a silica membrane for DNA purification requires elution of nucleic acid.[11] Tests performed by Elgort *et al.* using aluminium oxide membrane (AOM) aimed to integrate cell lysis and PCR in one chamber.[12] LIMBS as single step cell lysis and DNA isolation method provides rapid DNA release and the removal of denatured proteins from pathogens at the same time. Furthermore, we fabricated a portable sample

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preparation device and microchip using a small laser diode and magnet holder to use the same microchip for sample preparation and real-time pathogen detection in a PCR machine without changing the solution. Here we demonstrate simple and rapid DNA extraction and real-time detection of pathogens in a single chamber of a microchip within 32 minutes without channels, pumps or valves.

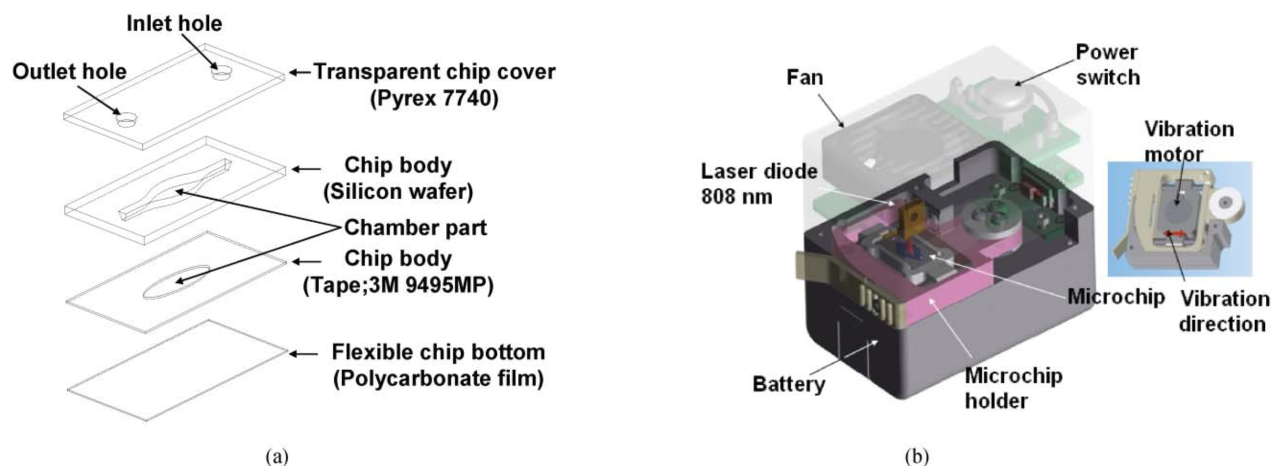
2. Materials and methods

2.1 Images of live and dead cells with magnetic beads

To check the effect of the laser power on the cell lysis, we separated and counted live and dead cells in the sample solution before and after the laser irradiation. A 9 μl sample of *E. coli* (1×10^5 cells μl^{-1}) was mixed with 1 μl of magnetic beads (100 μg μl^{-1}, 7–12 $\times$ 10^7 beads μl^{-1}) and stained using the Live/Dead® BacLight™ Bacterial Viability kit (L7012, Molecular Probe, USA) according to the procedure recommended by the suppliers before and after laser irradiation (laser output power: 1 W). Images were captured with a microscope (Eclipse TE 300, Nikon, Japan) equipped with a fluorescence filter cube (450 to 490 nm excitation filter, 500 nm dichroic mirror, and 515 nm long pass emission filter) and a Nikon D100 digital camera, using a 100× oil immersion objective lens.

2.2 Fabrication of microchip and portable laser induced sample preparation device

Microchips with a chip size of 7.5 mm × 15 mm and 10 μl sample volume were fabricated using silicon, glass, polycarbonate film, and double-coated tape (9495MP, 3M, MN, USA) in order to measure the temperature of solution and perform laser-induced sample preparation (Fig. 1a). The fabrication process for the microchip used for laser-induced sample preparation consisted of two photolithography steps and a bonding step with polycarbonate film using double-coated tape. A glass wafer with a diameter of six inches and a thickness of 500 μm was cleaned and laminated by a BF410 film photoresist. The photoresist was patterned by photolithography to form holes with a diameter of 1.5 mm for inlet and outlet sample passages. Holes were formed on the glass wafer by a sand blast technique. The silicon wafer was a double-sided polished silicon substrate with a diameter of six inches and a thickness of 680 μm. Chambers were formed on



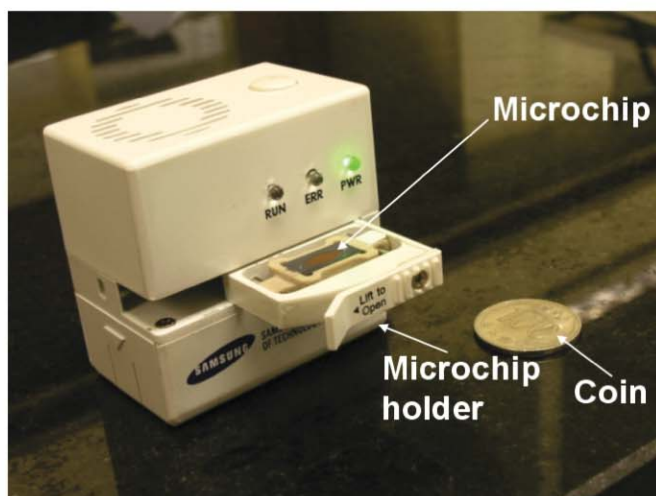


Fig. 1 Portable device for LIMBS and the single-chamber real-time detection of pathogens. (a) Schematic diagram of a microchip for temperature measurement. (b) Schematic diagram of a hand-held type LIMBS using small laser diode and microchip holder with vibration motor. (c) Image of hand-held type sample preparation device (58.3 × 57.9 × 37.0 mm, 148 g) with a small laser diode.

the silicon wafer by a sand blast technique due to cost concerns. The silicon wafer was bonded with the glass substrate using an anodic bonding technique. For optimization of sample loading, a streamline form shaped reaction chamber of 10 μl sample volume was designed, and Sigmacoat® (Sigma-Aldrich, MO, USA) was coated on the sand blasted surface of the silicon wafer to change the hydrophobicity. Polycarbonate film with a thickness of 100 μm was then bonded to the silicon wafer using double-coated tape with a thickness of 150 μm.

The temperature of the sample in the chamber of the microchip was measured by a thermocouple (K type, Omega) with a data acquisition system (34970A, Agilent, CA, USA). The thermocouple was inserted into the chamber very carefully, and then polycarbonate film was bonded to the silicon wafer using double-coated tape.

Microchips for the possibility of pathogen detection in one chamber (4 μl) without exchanging the buffers and reagents for PCR were fabricated using silicon and glass. The silicon wafer with a thickness of 500 μm was etched to a depth of 200 μm by a silicon wet etching solution (25% TMAH) to form the microchamber with a volume of 4 μl. The wet etched silicon wafer was bonded with the glass substrate using an anodic bonding technique.

A hand-held type sample preparation device was fabricated using a high power laser diode (L8446-72, L8828-72 Hamamatsu Photonics, Japan) in each experiment, while the microchip was vibrated by a coin-type vibration motor (DMJBRK20X, Samsung electro-mechanics, Korea). The energy source of the device is a lithium ion battery pack (7.4 V, 680 mA h, EN-EL1, Nikon, Japan). A microprocessor (PIC16F73) is used to regulate the laser power, control the vibration motor, and count down the operation time. Setting values can be downloaded from a PC and stored in the EEPROM of the device by RS-232 communication. A schematic diagram and an image of the hand-held type sample preparation device (58.3 × 57.9 × 37.0 mm, 148 g) are shown in Fig. 1b,c.

Both inlet and outlet holes were sealed with optically transparent adhesive tape (Applied Biosystems, CA, USA) after loading the sample solution with magnetic beads.

2.3 Bacterial strain and quantification of bacterial genomic DNA

E. coli strain BL21 and *Streptococcus mutans* (ATCC# 35668) were cultured at 37 °C with vigorous aeration in brain heart infusion (BHI) media to an exponential phase (OD_{600} = 0.5–1.0). *Staphylococcus epidermidis* (ATCC#14990→12228) was cultured at 37 °C with vigorous aeration in Nutrient Agar (NA) media to an exponential phase (OD_{600} = 0.5–1.0). The bacterial cells were harvested by centrifugation and washed twice with 3 ml of phosphate-buffered saline (PBS). The cells were resuspended in PBS (cell density; 1×10^5 cells μl^{-1}). For bacteria lysis, 9 μl of bacterial cells and 1 μl of concentrated magnetic beads (100 μg μl^{-1}, 7–12 × 10^7 beads μl^{-1}, Dynabeads® MyOne™ Carboxylic Acid, DYNAL, Norway) were mixed in a microchip (SAIT, Korea) and placed in a chip guide module (AMITECH, Korea). The hand-held type sample preparation device was used a high power laser diode (L8446-72, Hamamatsu Photonics, Japan) in our experiments, while the microchip was vibrated by a coin-type vibration motor (DMJBRK20X, Samsung electro-mechanics, Korea).

In order to compare the efficiency of DNA release by laser method with the efficiency by other known conventional methods, we prepared genomic DNA (from 0.9×10^5 cells, equivalent to the number of cells used for each laser lysis) using the Qiagen DNA extraction kit (Cat 13323, Blood & Cell Culture DNA Mini Kit, Qiagen, Germany) with lysozyme treatment for 30 minutes and a pressure cell lysis device (Quantity/shot1, Constantsystems, Germany) according to the procedure recommended by the suppliers, with 40 kpsi for Gram-positive bacterial cells and 15 kpsi for Gram-negative bacterial cells. We also prepared *E. coli* genomic DNA (from 0.9×10^5 cells, equivalent to the number of cells used for each laser lysis) using a boiling method for 5 minutes at 95 °C.

To monitor bacterial lysis and to quantify the amount of DNA released from the lysed cells, we used Agilent Bioanalyzer2100 (Agilent Technologies, Palo Alto, CA) followed by polymerase chain reaction (PCR) amplification. The following primers were used for PCR: primer A, 5′-CCCAGACTCCTACGCGAGGC-3′; primer B, 5′-GTA-TTACCGCA ACTGCTGGCAC-3′. These are complementary to each end of a gene encoding the 16S ribosomal RNA, allowing the amplification of its entire coding region during PCR. *E. coli* PCR amplifications were carried out for 25 cycles and PCR amplifications for Gram-positive bacterial cells, *Streptococcus mutans* and *Staphylococcus epidermidis*, for 30 cycles (95 °C for 1 minute to pre-denature, 95 °C for 5 seconds to denature, 60 °C for 13 seconds to anneal, and 72 °C for 15 seconds to extend, and 72 °C for 1 minute for additional extension) using Taq polymerase (Solgent Co. Ltd, Korea). After amplification cycles were completed, a melting curve was acquired by slowly heating (0.1 °C s^{-1}) the sample from 60 to 90 °C. The PCR was performed by LightCycler® (Roche Diagnostics Corporation, IN, USA) with a total volume of 20 μl reaction mixture containing 1X FastStart DNA Master SYBR (Roche Diagnostics Corporation, IN, USA), 0.25 μM forward and reverse primers (Genotech, Korea), 4 mM $MgCl_2$ (Roche Diagnostics Corporation), and D.W. (PCR grade, Roche Diagnostics Corporation, IN, USA).

2.4 Preparation of beads for effect of surface charge of magnetic beads and material of beads

To see the effect of the surface charge and material properties of the beads, carboxylic acid-terminated polystyrene magnetic beads (1.02 μm, 1 μl, 100 μg μl^{-1}, 7–12 × 10^7 beads μl^{-1}, Dynabeads® MyOne™ Carboxylic Acid, DYNAL, Norway), amine-terminated polystyrene magnetic beads (1.5 μm, Bangs Laboratories Inc., IN, USA), polystyrene beads (4.16 μm, Bangs Laboratories Inc., IN, USA), and silica beads (3.0 μm, Bangs Laboratories Inc., IN, USA) were prepared. Bacterial cells (9 μl), prepared as mentioned above, were mixed with beads (1 μl) in a microchip (10 μl, SAIT, Korea) placed in a chip guide module (AMITECH, Korea).

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2.5 Quantification of HBV by the real-time PCR and protein quantification

30 μl of hepatitis B virus (HBV) (1×10^7 pfu), was mixed with 60 μl of human serum and magnetic beads (30 μl, 2×10^9 beads ml^{-1}, Dynabeads® M-270 Carboxylic Acid, DYNAL, Norway) were mixed in a vial (C4010-15, Young-Wha Scientific Co. Ltd, Korea) with a vial guide (AMITECH, Korea) for rapid cell lysis and large sample volume treatment. A high power laser beam at 808 nm (13.8 W of laser output power) was fired at the sample for the time indicated using fiber-coupled laser systems (HLU25F100-808, LIMO, Germany), while the vial was agitated by a vortexer.

HBV serotype ayw was kindly provided by Professor Wang-Shik Ryu (Department of Biochemistry, Yonsei University, Korea). To monitor cell lysis and to quantify the amount of DNA released from HBV, we used real-time PCR, and the PCR product was quantitated again using Agilent Bioanalyzer2100 for verification. The following primers were used for the real-time PCR: Primer C forward: 5′-AGTGTGGATTCGCACTCCT-3′, primer C reverse: 5′-GATTCTTCTTCTAGGGGACCTG-3′. The primer set C for the core region detection was designed, originally by Chen *et al.*,[13] against a highly conserved region among 25 published HBV genome sequences in GenBank and EMBL, representing genotypes A–F. The primer set C was designed to amplify a 118 bp fragment in a core region. PCR amplification was carried out using Taq polymerase (Solgent Co. Ltd, Korea) for 40 cycles (50 °C for 10 minutes and 95 °C for 1 minute to pre-denature, 95 °C for 5 seconds to denature, 62 °C for 15 seconds to anneal and extend). After amplification cycles are completed, a melting curve was acquired by slowly heating (0.1 °C s^{-1}) the sample from 60 to 90 °C. The PCR was performed by LightCycler® (Roche Diagnostics Corporation, IN, USA) with a total volume of 20 μl reaction mixture containing 1X FastStart DNA Master SYBR (Roche Diagnostics Corporation, IN, USA), 0.5 μM of forward and reverse primers (Genotech, Korea), 5 mM $MgCl_2$ (Roche Diagnostics Corporation), 0.01 U μl^{-1} Uracil-N-glycosylase (Solgent, Korea). The LightCycler® capillaries were first incubated at 50 °C for 10 minutes in order to destroy the potential carry-over contamination using the Uracil-N-glycosylase (UNG), after inactivating the UNG enzyme by heat treatment at 95 °C for 1 minute.

SEM images of magnetic beads were analyzed using the FE-SEM (S-4500, Hitachi High-Technologies Corporation, Japan) to show the increased binding of proteins to the beads after lysis. Proteins bound to the magnetic beads after laser radiation were quantified by SDS-PAGE (polyacrylamide gel electrophoresis) and coomasie blue staining for the total protein quantification.

2.6 Optimization of key factors in LIMBS and sample preparation of HBV in a microchip

To optimize LIMBS, we checked the effect of three key factors. For accurate quantification, we checked the crossing point value (Cp) of DNA amplification by LightCycler® (Roche Diagnostics Corporation, IN, USA), as well as the amplified DNA concentration by Agilent BioAnalyzer 2100. We used the amount of DNA released after boiling at 95 °C for 5 minutes as a reference. We added a 9 μl *E. coli* (1×10^5 cells μl^{-1}) sample with 1 μl magnetic beads (50 μg μl^{-1}, 7–12 $\times 10^7$ beads μl^{-1}) to the microchip, and the laser was fired with different laser output powers as indicated, with vibration (2 V motor power). We also checked the effect of the amount of magnetic beads. We added different amounts of beads to the sample solution (0 to 9 μg in 10 μl of sample solution) in the microchip, and then irradiated with 1 W of laser output power for 40 seconds, with 2 V of vibration motor power.

Finally, we checked the effect of the external vibration for the sample chip. We added a 9 μl *E. coli* (1×10^5 cells μl^{-1}) sample with 1 μl magnetic beads (100 μg μl^{-1}) in the microchip, and the microchip was vibrated with the indicated vibration motor power, using 1 W of laser output power.

For sample preparation of HBV, 2 μl of HBV (1×10^7 pfu), 0.4 μl of human serum, 1.2 μl of PBS and 0.4 μl magnetic beads were mixed in the microchip (4 μl).

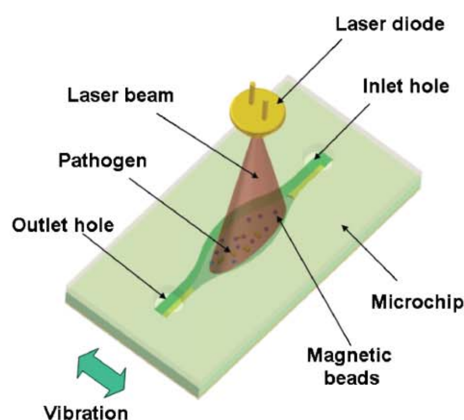
2.7 One-chamber TaqMan real-time PCR analysis after laser-bead lysis

E. coli BL21 was premixed with 0.25 U μl^{-1} Taq polymerases and 100 μg of 1 μm magnetic beads in the following PCR mixture: 75 mM Tris-HCl (pH 9.0), 15 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 1 mg ml^{-1} BSA, 250 μM dNTP mixture, a pair of 1 μM PCR primer solutions (5′-TGTATGAAGAAGGCTTCG-3′ and 5′-AAAGGTATTAACTTTAC TC-3′) and 0.4 μM TaqMan probes (FAM-5′-TGTATGAAGAAGGCTTCGG-GTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAG-TAAAGTTAATACCTTT -3′-TAMRA). After 1 W laser irradiation for 40 seconds, magnetic beads were driven to one side of the microchip by putting the microchip into a magnet-holder, and the real-time PCR was performed directly after transferring the microchip to the GeneSpector® Micro PCR (SAIT, Korea). We used 1 minute at 95 °C to denature the DNA completely, and 40 cycles of 10 seconds at 95 °C, 10 seconds at 50 °C and 10 seconds at 72 °C.

3. Results and discussion

3.1 Cell lysis by laser-irradiated magnetic bead system (LIMBS)

Pathogens and magnetic beads were loaded into a microchip placed in a chip guide module. To lyse pathogens, a high power laser beam at 808 nm was applied to the microchip concurrently with vibration provided by a portable motor (Fig. 2a). The 808 nm laser wavelength was selected based on the absorption coefficient of this wavelength in water.[14,15] Because the absorption coefficient of 808 nm laser in water is 0.021773 (cm^{-1}),[14] most of the laser beam is transmitted through the water and reaches the magnetic beads with only a minute amount of absorption. A visible laser beam is also applicable, but a high power portable laser diode is not readily available. An IR laser is not suitable for our study because the absorption coefficient of IR wavelength in water is very high; most of the IR laser energy would be absorbed in the water. A UV laser beam is not good for cell lysis and DNA purification because UV irradiation causes DNA damage by producing



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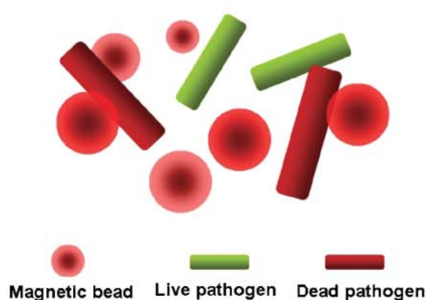

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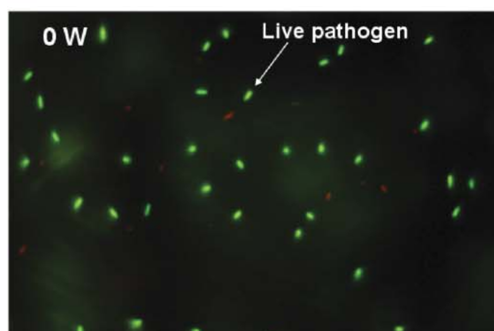

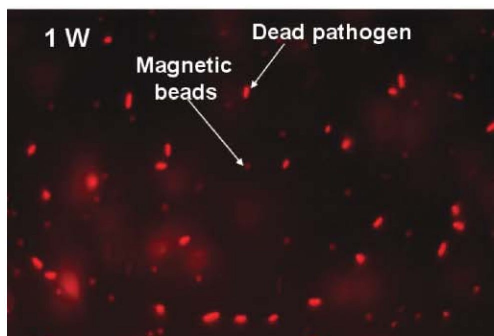

(c)

thymine dimers as the major photoproduct.[16] Thus, we employed a continuous laser diode with a wavelength of 808 nm.

A laser was fired on to the chamber area in a vibrating microchip which contained pathogens and magnetic beads (Fig. 2a). Cell lysis is most likely caused by the combination of the heat shock and the mechanical shock delivered by the magnetic beads. Highly heated magnetic beads bump against the cells, transfer heat and presumably disrupt the cell walls and membranes (Fig. 2b). All of the green colored cells (live) turned to red (dead) after laser irradiation in the presence of microbeads (Fig. 2c), suggesting that the laser-irradiated magnetic beads lysed whole bacteria effectively. The survival rate of cells decreased with increased laser power and exposure time. In the absence of beads, the survival rate was not changed after laser irradiation (laser output power: 1 W).

3.2 Development of LIMBS for rapid DNA isolation

Given that the bacterial cells were lysed efficiently by LIMBS, we next tried to develop a novel concept for rapid DNA isolation using LIMBS. As shown in Fig. 3a, we developed the application of LIMBS as a single step detection method for rapid DNA release and the removal of denatured proteins at the same time.

To increase the efficiency of DNA release and prevent the binding of DNA to the beads during the lysis, we first compared two beads having different surface charges: the carboxylic acid-terminated polystyrene magnetic beads (CPM) having negative charges on the surface, and amine-terminated polystyrene magnetic beads (APM) having positive charges on the surface.

To compare the efficiency of DNA release, we quantified the amount of released DNA by Agilent Bioanalyzer2100 after laser irradiation. The laser was fired with 1 W of laser output power at a 10 μl sample in the microchip, with 4 V of vibration power.

The amount of released DNA using CPM (10.77 ng μl^{-1}) was greater than the amount by APM (0.11 ng μl^{-1}) (Fig. 3b). This is due to charge repulsion by the negative charge between the carboxyl groups on CPM and DNA. In other words, in the case of APM, the DNA released after the cell lysis binds to the microbeads due to the charge attraction to the positive charge of the amine functional groups on the surface of the beads. We hypothesized that there might be a correlation between the temperature increase of the beads and the DNA release after

Fig. 2 Laser-Irradiated Magnetic Bead System (LIMBS) designed for the rapid lysis of bacterial cells. (a) Schematic diagram of the microchip and the laser irradiation. A high power laser beam at 808 nm was irradiated to the microchip for 40 seconds using a laser diode to lyse cells. (b) Possible mechanism of the cell lysis by LIMBS. Strongly heated micro magnetic particles transfer heat to cells and directly impact cells by laser ablation. (c) Fluorescent microscopy of bacterial cells showing before laser irradiation (A) and quick lysis after laser irradiation (B). An *E. coli* (1×10^5 cells μl^{-1}) sample was stained using the Live/Dead® BacLight™ Bacterial Viability kit (L7012, Molecular Probe, OR, USA) according to the manufacturer's recommendation to check the viability of cells. The survival rate of cells dropped to 0% with 1 W laser irradiation.

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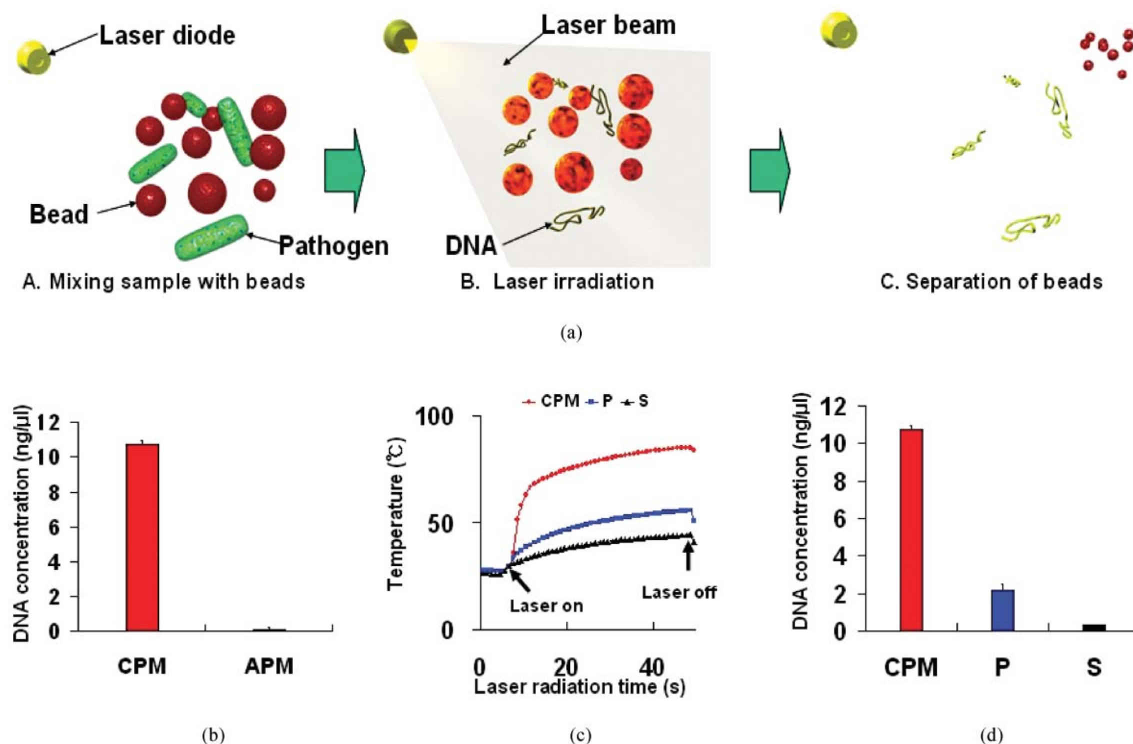
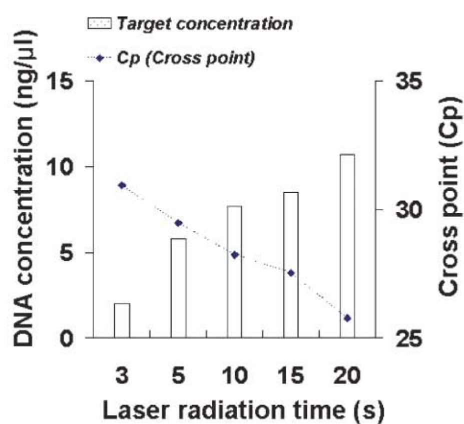


Fig. 3 Single step DNA isolation by LIMBS. (a) Schematic representations show the principle of laser irradiation and subsequent DNA isolation. Biological samples are mixed with microbeads (left). When the laser is fired, heated microbeads lyse cells and the denatured proteins are adsorbed to the surface of the beads while DNA remains in the solution (middle). When the magnetic microbeads are removed, sample solution is ready for further analysis such as PCR amplification (right). (b) DNA concentration after the laser irradiation in the presence of bacteria. The amount of isolated DNA is dependent on the surface charge. Three replicates of each condition were tested, and the error bars represent the standard deviation of the mean. CPM: carboxylic acid-terminated polystyrene magnetic beads, APM: amine-terminated polystyrene magnetic beads. (c) Temperature change of sample solution in a chamber of the microchip after laser irradiation is dependent on the material of the microbeads. Temperature was measured using a thermocouple. (d) The amount of isolated DNA is dependent on the material of the microbeads. Three replicates of each condition were tested, and the error bars represent the standard deviation of the mean. P: polystyrene microbeads, S: silica beads.

the same dose of laser irradiation depending on the material properties. To prove this, we measured the temperature of the sample solution inside the microchip with different beads using a thermocouple (Fig. 3c) and quantified the amount of released DNA (Fig 3d). The temperature of the sample solution containing CPM increased rapidly because of the high heat capacity of the iron component and good absorbance of the laser beam. With CPM, the cell lysis was efficient and 10.77 ng μl^{-1} of DNA was released. In contrast, the temperature of the sample solution containing polystyrene beads (P) increased slowly because of the low heat capacity and low absorbance of the laser beam, as evidenced by only 2.17 ng μl^{-1} DNA being released. With silica beads (S), the temperature increased very slowly because of poor absorbance of the laser beam. The resulting target DNA release was very inefficient (0.34 ng μl^{-1}).

Next, we studied how DNA is separated from the proteins after the cells are lysed in LIMBS. We used hepatitis B viruses (HBV) mixed with 50% human serum. Because of the serum proteins, hepatitis B viruses are not detectable by simple boiling and PCR. Interestingly, we observed efficient detection of viruses by LIMBS and PCR in the presence of human serum proteins (Fig. 4a). We hypothesized that the magnetic beads remove the substances that interfere with the PCR amplification, and we checked the magnetic beads by scanning electron microscopy (SEM) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) after laser irradiation.

When we observed the beads by SEM after the laser irradiation on the sample mixed with beads, HBV and serum proteins, we found that the surface shapes of the beads are changed in the sample solution, suggesting that the denatured proteins and cell debris are adsorbed on to the polystyrene surface of the magnetic beads (Fig. 4b). This was confirmed quantitatively by the biochemical analysis of the beads using SDS-PAGE after laser irradiation for indicated times on the sample containing viruses in the human serum (Fig. 4c). Although we observed minor nonspecific adsorption of proteins to the beads without the laser irradiation (column 2), there was a massive binding of proteins to the beads, especially after laser irradiation for 10 seconds or more (columns 5, 6, 7). These results suggest that the magnetic beads removed a substantial amount of denatured proteins from the sampling solution and significantly improved the efficiency of the PCR analysis by lowering the limit of detection, reducing the time of DNA extraction and increasing the signal amplitude.



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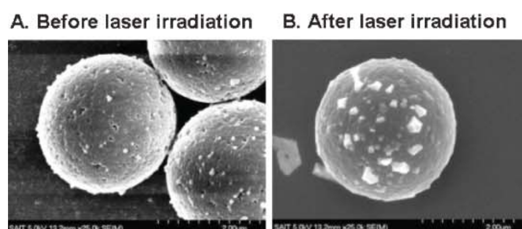

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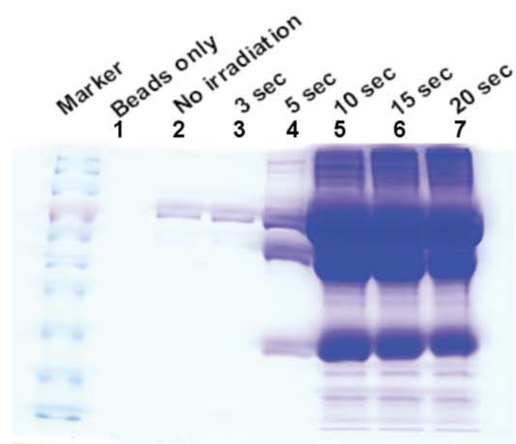

(c)

Fig. 4 Laser radiation released hepatitis B virus DNA efficiently in the presence of magnetic beads. (a) As the laser irradiation time is increased, more DNA is released, as measured by the change of cross point of real-time PCR and electrophoresis after the laser irradiation. (b) SEM image of micro magnetic beads before (A) and after (B) the laser irradiation in the presence of hepatitis B virus with human serum. (c) SDS-PAGE analysis on the magnetic microbeads showing the massive adsorption of proteins after laser irradiation in the presence of hepatitis B virus with human serum. Beads were washed after the laser irradiation for the indicated time. Marker column: protein molecular weight markers: 170, 130, 100, 72, 55, 40, 33, 24, 17, 11 kD from the top. Column 1: beads only; 2: no laser irradiation; 3: 3 seconds laser irradiation; 4: 5 seconds laser irradiation; 5: 10 seconds laser irradiation; 6: 15 seconds laser irradiation; 7: 20 seconds laser irradiation.

3.3 Key factors and the limit of detection of LIMBS

To optimize LIMBS, we checked the effect of several factors that might influence the efficiency of the sample lysis and DNA release after laser irradiation. We used the amount of DNA released after boiling at 95°C for 5 minutes as a reference. The crossing point (Cp) value decreased as we raised the laser output power, suggesting that the laser power is a critical factor (Fig. 5a). Cp is defined as the cycle number at which the fluorescence passes the fixed threshold. It has been reported that 10-fold differences in the template DNA concentration results in 3.32 cycle differences in Cp when PCR efficiency is 100%.[13,17] We selected 1 W of laser output power to design the portable device with minimum consumption of electric power, since it showed sufficiently higher DNA release than boiling.

As the laser radiation power was increased, the sample temperature increased more rapidly (Fig. 5b). In particular, the sample temperature immediately increased above 65°C, the cell inactivation temperature, after laser irradiation above 1 W for a few seconds. We selected 40 seconds with 1 W of laser output power as a standard laser irradiation on to the microchip, which was enough to lysis pathogens efficiently.

Next, we checked the effect of the external vibration for the sample chip. When we increased the power of the vibration motor, we observed more DNA release (Fig. 5c). We selected 4 V of vibration power for the portable device to be optimal with low laser output power (0.5 W of low laser output power was used because 1 W was too strong to see the vibration effect). At a vibration motor power over 4 V, the vibration motor was not stable and the vibration effect was saturated. Also, vibration of the microchip increased the laser-irradiated area, implying that the heated beads had more chances to collide with the targets. These results suggest that the DNA release is not just a result of simple thermal inactivation, but the lysis depends on transferring thermal and physical energy to the cell wall and membranes.

Finally, we checked the effect of the amount of magnetic beads. We added different amounts of beads to the sample solution and irradiated the sample with the laser. DNA release was increased with more beads. A bead density above 0.5 $\mu g\ \mu l^{-1}$ of sample solution showed efficient cell lysis and DNA release (Fig. 5d). We selected a bead density of 0.9 $\mu g\ \mu l^{-1}$ for the portable device to be optimized with the laser output power. Above the 0.9 $\mu g\ \mu l^{-1}$ bead concentration, the amount of released DNA was saturated (data not shown).

To determine the limit of pathogen detection by LIMBS, we added a different number of *E. coli*, and real-time PCR was performed after laser irradiation. The amplified PCR product was electrophoretically confirmed. We could detect even down to twenty *E. coli* cells μl^{-1} in a 10 μl sample volume. *E. coli* that had not been irradiated served as a negative control and yielded no significant target DNA peak (Fig. 5e).

3.4 LIMBS is superior to other conventional lysis methods

To see whether LIMBS is advantageous for detecting pathogens, we investigated the lysis efficiency of Gram-positive bacterial cells, which are known to be difficult to disrupt, with other commonly used methods. *Streptococcus mutans* and *Staphylococcus epidermidis* (cell density: 1×10^5 cells μl^{-1}) were mixed with magnetic beads, and we checked whether DNA was

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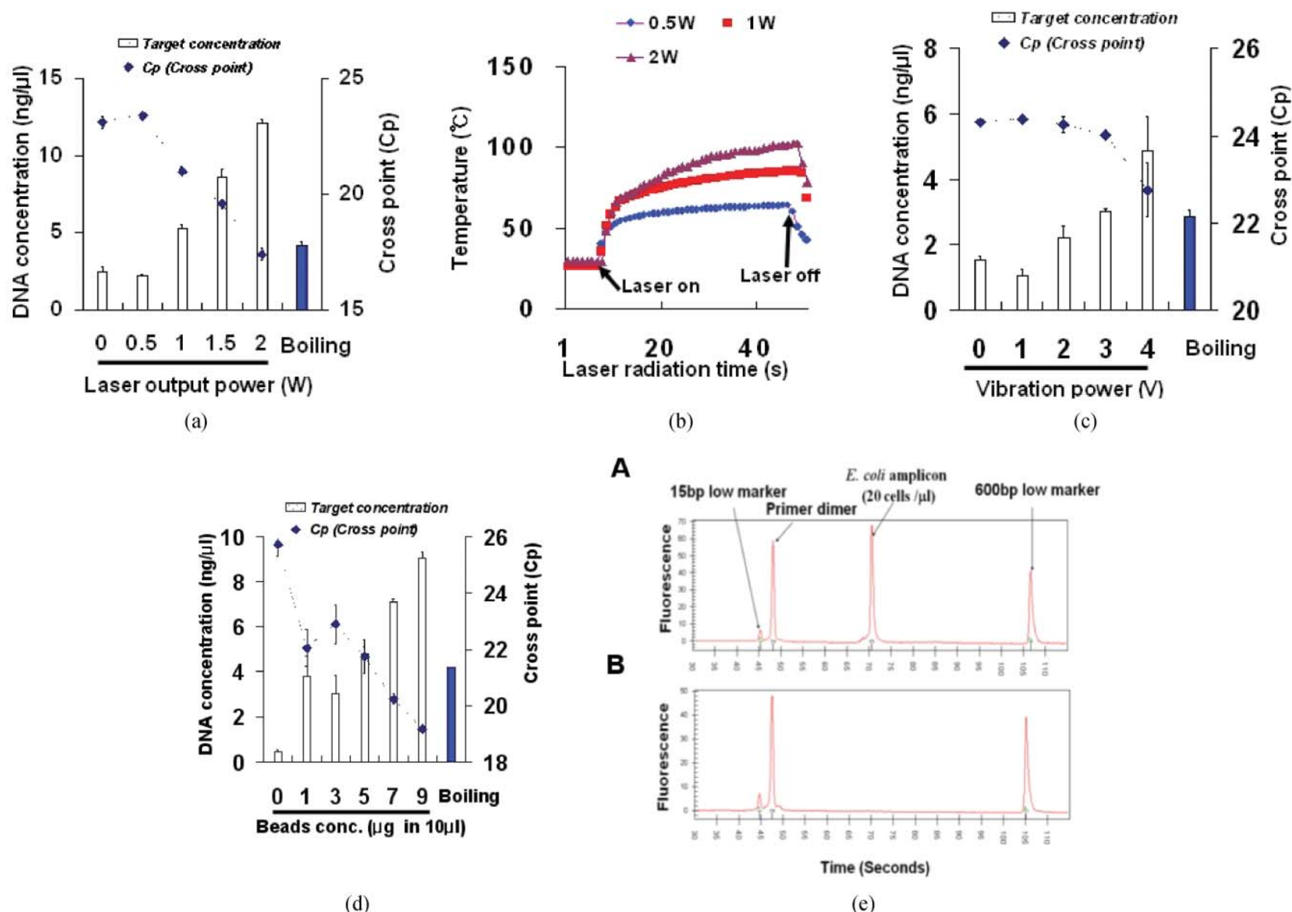


Fig. 5 Key factors for LIMBS. Results from the laser–microbeads technique were compared with boiling at 95 °C for 5 minutes in each experiment. Key factors for the efficiency of cell lysis were measured by real-time PCR and released DNA quantification. (a) Effects of the laser output power for efficient cell lysis. (b) Effects of the laser output power on the temperature increase in the microchip chamber. (c) Effects of vibration power for cell lysis. (d) Effects of magnetic beads concentration on *E. coli* cell lysis. (e) Limit of detection of LIMBS. 20 cells μl^{-1} of *E. coli* (equivalent to 100 fg genomic DNA) in 10 µl sample volume were detected after laser lysis and PCR amplification for 25 cycles (A). No laser irradiation as a negative control (B).

detectable by the real-time PCR after laser irradiation. In order to compare the efficiency of DNA release directly using the laser irradiation with other conventional cell lysis methods, we used Qiagen kits for cell lysis and purification of released DNA. We also compared the pressure cell lysis method. Sample preparation by LIMBS showed higher efficiency of DNA release from the same number of cells than other conventional cell lysis methods as measured by the change of Cp (Fig. 6a) and the released DNA concentration (Fig. 6b). Using hepatitis B virus (HBV) as a model for a viral pathogen, we also checked the detection specificity of LIMBS in the microchip (Fig. 6b). By the boiling method, we could not detect HBV DNA because of the substances in human serum that interfere with the PCR amplification.

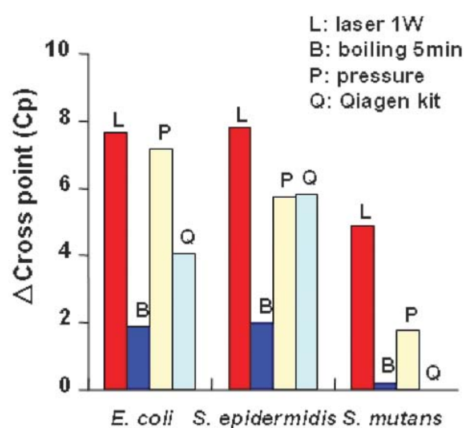
3.5 Subsequent real-time detection of pathogen using LIMBS in one microchip

After optimizing the LIMBS, we tested the possibility of real-time pathogen detection in one chamber (4 µl) without exchanging the buffers and reagents for real-time PCR after laser irradiation to the microchip. To do this, *E. coli* (2×10^4 cells μl^{-1}) was added to magnetic beads and premixed PCR reagents. The portable sample preparation device (58.3 × 57.9 × 37.0 mm, 148 g) was used to irradiate with 1 W of laser output power for 40 seconds (Fig. 1b,c). Real-time PCR (GeneSpector®, Micro PCR SAIT, Korea)[17–19] was performed after the magnetic beads were driven towards the magnet in the microchip cartridge (Fig. 7a) by the TaqMan assay method designed to recognize the 16S ribosomal RNA region of bacterial genome. Laser irradiation itself does not have significant effects on PCR reagents (data not shown). As shown in Fig. 7b, we tried four repetitions and obtained reproducible Cp values (N = 4, mean 25.322, standard deviation 0.646) after 40 cycles.

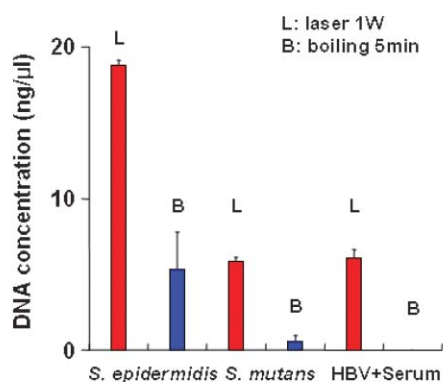
It is noteworthy that the cell lysis using the portable sample preparation device and the real-time detection in a micro-PCR device was performed using a single microchip without changing reagents and that the whole detection process took less than 32 minutes.

4. Conclusion

Recently, lasers have been applied to cell disruption and are being tried for LOC application,[20] but these trials were not

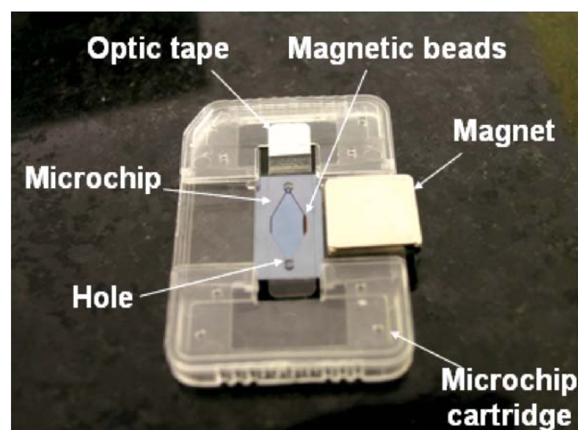


(a)

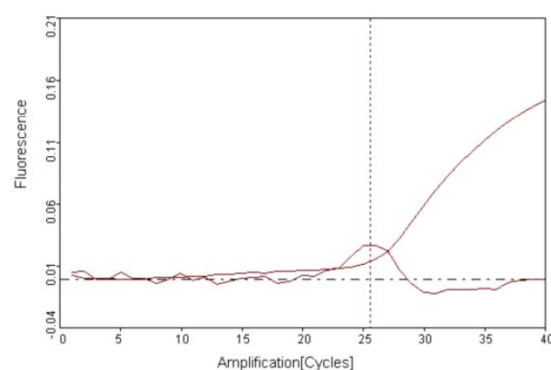


(b)

Fig. 6 Efficient DNA release by LIMBS. (a) Comparison of LIMBS with other conventional cell lysis methods. ΔCp (difference of cross point value of lysis and supernatant) comparison with other conventional methods. (b) DNA was isolated by various methods as indicated and quantified after the PCR amplification. Efficient detection of hepatitis B virus (HBV) by LIMBS. LIMBS cell lysis is superior to conventional cell lysis methods.



(a)



(b)

Fig. 7 The single-chamber real-time detection of pathogens. The total process of pathogen detection, from the cell lysis to the real-time polymerase chain reaction (PCR), was performed successfully in one chamber of the microchip in a portable LIMBS device. (a) Image of the microchip with magnet bonded microchip cartridge after laser irradiation. Magnetic beads were attracted toward the magnet completely in the microchip. (b) Detection of 2×10^4 *E. coli* cells μl^{-1} in one chamber premixed with PCR reagents and Taq polymerase before laser irradiation. The DNA was adequately amplified with a total of 40 cycles by TaqMan assays. The total process was performed in one chamber within 32 minutes using a portable LIMBS device.

practical and have many problems. Given that the most of LOC platforms that have been published so far not only have external pumps and many valves but also very complex microfluidic channels like a spider's web,[21,22] multiple chambers on a microchip, a large size and high power consumption, our novel LIMBS is a practical breakthrough for a true LOC device.

Here, we have developed Laser-Irradiated Magnetic Bead System (LIMBS) and demonstrated rapid sample preparation using a small size laser diode and magnetic beads in a chamber of a microchip with a portable LIMBS device. This provided efficient cell lysis and DNA isolation for various pathogen detections. We also realized this system in a disposable microchip as a portable sample preparation device with vibrating magnetic beads present in cell suspension that caused rapid cell lysis and DNA purification when the magnetic beads were irradiated by a laser beam. We studied the basic mechanism of the cell disruption by LIMBS to optimize the conditions for the cell lysis and DNA release.

It is noteworthy that the Gram-positive bacterial cells (*Streptococcus mutans*, *Staphylococcus epidermidis*) as well as HBV mixed with human serum were easily disrupted within 40 seconds in the microchip. Furthermore, we fabricated a portable sample preparation device and microchip using a small laser diode and magnet holder to use the same microchip for sample preparation and real-time pathogen detection in a PCR machine without changing the solution. We demonstrated that this method provides a better DNA yield than other conventional methods, as quantified by Agilent Bioanalyzer2100 and real-time PCR.

In summary, the easy and efficient cell lysis that releases DNA with minimum interference on PCR amplification by the LIMBS provides a novel real-time detection method

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well-suited to LOC applications and other applications related to DNA analysis.

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