LoMA-B: A rapid and simple lab on a chip system based on single-channel bisulfite conversion for DNA methylation analysis

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Abstract

Miniaturized Lab on a Chip (LOC) systems have been developed for genetic and epigenetic analyses in clinical applications because of advantages such as reduced sample size and reagent consumption, rapid processing speed, simplicity, and enhanced sensitivity. Despite tremendous efforts made towards developing LOC systems for use in the clinical setting, the development of LOC systems to analyze DNA methylation, which is an emerging epigenetic marker causing the abnormal silencing of genes including tumor suppressor genes, is still challenging because of the gold standard methods involving a bisulfite conversion step. Existing bisulfite-conversion based techniques are not suitable for clinical use due to their long processing-time, labor-intensiveness, and the purification steps involved. Here, we present a Lab-on-a-Chip system for DNA Methylation Analysis based on Bisulfite conversion (LoMA-B), which couples a sample pre-processing module for on-chip bisulfite conversion and a label-free, real-time detection module for rapid analysis of DNA methylation status using an isothermal DNA amplification/detection technique. The methylation status of the *RARβ* gene in human genomic DNA extracted from MCF-7 cells was rapidly analyzed by the LoMA-B system within 80 min compared to conventional MS-PCR (3 to 24 h). Furthermore, the LoMA-B system is highly sensitive and can detect as little as 1% methylated DNA in a methylated/unmethylated cell mixture. Therefore, the LoMA-B system is an efficient diagnostic tool for the simple, fast, and quantitative evaluation of DNA methylation patterns for clinical applications.

Introduction

A microfluidic lab on a chip (LOC) with a miniaturized and automated system to monitor cancer biomarkers has been developed in the last two decades. It has enabled simple and rapid micro-scale analysis for clinical applications by taking advantages of its high throughput, highly sensitive process, and allowing a reduction in sample size and reagent consumption.[1–2] In addition, elastomers, the materials that make up the devices and make them more biocompatible at a low-cost, have evolved from traditional silicon and glass.[1–3] Because of these advantages, a myriad of LOC systems for genetic testing of diseases with related biomarkers have been developed that integrate advanced detection features including capillary array electrophoresis (μ-CAE), nanowire-based transistors,[4] carbon nanotubes,[5] silicon photonic based sensors,[6–7] electrochemical impedance spectroscopy,[8] and solid state nanopores.[9]

While a myriad of LOC systems for genetic analysis have been developed in order to meet the unmet needs of clinical setting, LOC system for epigenetic analysis has been relatively unexplored due to it requires complex steps.[10–11] Thus, the development of LOC system based on epigenetic analysis remains challenging. Epigenetic modifications of the genome, such as DNA methylation, histone modification, and microRNA-associated gene silencing, are known to be associated with cancer causation and progression by regulating gene expression without altering the gene nucleotide sequence.[12–13] Particularly, abnormal silencing of tumour suppressor genes results from DNA hypermethylation of the promoter region, potentially leading to cancer development and progression.[12–13] Recently, DNA methylation has been recognized as an attractive biomarker for early cancer diagnosis, identification of effective treatment, and prediction of response to chemotherapeutic agents by several international consortiums including the Human Epigenome Consortium (HEC) and NIH Roadmap Epigenomics Consortium.[14–15] The aim is to develop large-scale mapping of human DNA methylation to determine the necessity of epigenetic analysis of cancer for research and clinical use. Hence, the development of a reliable and robust diagnostic tool based on the LOC system for rapid and simple analysis of DNA methylation status would be desirable for DNA methylation testing in research and clinical applications.

Amongst numerous DNA methylation testing methods, methylation specific (MS)-polymerase chain reaction (PCR) is one of the most widely used techniques based on bisulfite conversion.[16-

[17] The bisulfite conversion approach exploits the sequence difference induced by a chemical reaction which converts unmethylated cytosine to uracil while methylated cytosine remains intact. Although bisulfite conversion-based methods enable the determination of genome-wide DNA methylation status with high accuracy, they have not been previously applied to miniaturized LOC systems because of limitations including complex steps, long processing-time, and labor-intensive processing. In addition, false results (negative or positive) of DNA methylation analysis might be caused by long incubation times at a high temperature.[18–19] Meanwhile, alternative techniques ("bisulfite-free") have been devised to bypass the constraints of bisulfite conversion-based techniques for the analysis of DNA methylation status. Most techniques are based on endonuclease digestion,[20–23] electrochemical sensor-enzyme digested target hybridization,[24] affinity enrichment,[25-26] nanowire field effect-based biosensor anti-5-methylcytosine antibodies,[27] solid-state nanopores combined with labeling of DNA with methyl-CpG-binding domain (MBD) protein,[28] and nanofilm.[29] These techniques are relatively easy to adapt to a biosensor system and are successful at providing qualitative and quantitative analysis of DNA methylation status. However, compared to the bisulfite conversion-based technique, the aforementioned techniques result in low sensitivity and a smaller coverage of the sequence because of the enzyme that recognizes specific sequences only for cleavage. Thus, despite the limitations of the bisulfite conversion technique, MS-PCR is still the most frequently used to obtain comprehensive information about the DNA methylation status by using a next generation sequencer such as the Illumina Infinium assay.[30-31] Additionally, PCR based techniques including methylation-sensitive high resolution melting (MS-HRM) and MethyLight have been developed for sensitive and robust assessment of methylation.[32-35] Nevertheless, applying these techniques in clinical setting is still challenging because of the large number of chemical treatment steps involved, laborious and time-consuming processing, and potential sample loss and contamination during transferring samples between each step, which is inevitable when off-chip reactions are performed.[36–38] Therefore, a miniaturized LOC system based on on-chip bisulfite conversion holds great potential as a powerful tool for highly accurate DNA methylation profiling in the clinical setting.

Here, we developed a simple and rapid Lab on a chip system for DNA Methylation Analysis based on Bisulfite conversion (LoMA-B). The system integrates a single microfluidic channel

based on bisulfite conversion as a sample pre-processing module and an isothermal solid-phase amplification/detection (ISAD) as a detection module. First, the sample pre-processing module for single-chip bisulfite conversion was fabricated with multi-layered polymethylmethacrylate (PMMA) using a laser cutting machine for rapid prototyping,[23,39] which enables low-cost, simple, and fast fabrication. This module of the LoMA-B system offers highly reproducible on-chip-based bisulfite conversion for analysis of DNA methylation status within 50 min compared to the conventional bisulfite conversion method, which requires over 16 h. Second, the ISAD technique as a detection module for the LoMA-B system was employed after the sample pre-processing module for rapid DNA amplification and detection in a label-free and real-time manner. The ISAD technique uses a silicon biophotonic sensor for simultaneous DNA amplification/detection within 10–30 min, has been validated in clinical applications involving cancer and infectious disease, and allows for highly sensitive, specific, and low-cost fabrication because of its use of complementary metal-oxide semiconductor (CMOS) technology.[40–42] Finally, we demonstrated that the entire LoMA-B system analyzed the methylation status of retinoic acid receptor beta (*RARβ)* gene obtained from MCF-7 cells, a human breast cancer cell line,[43] within 80 min (total analysis time) compared with the 3–24 h required for MS-PCR. Furthermore, the LoMA-B system was able to detect the methylated DNA in a sample containing only 1% methylated DNA in a mixture of unmethylated DNA. The DNA methylation discovered in the *RARβ* gene is associated with several human cancers, and its detection is required to improve the effectiveness of clinical treatment.[43] The current system allows for the rapid (80 min) and simple detection of methylated DNA and is superior to methylation-specific polymerase chain reaction (MS-PCR) in its ability to analyze the methylation status of genes in human cancer cells. Therefore, the system described in this study is useful not only as a DNA methylation analysis system but also as a combination technique using existing PCR and next generation sequencing (NGS) methods for various research and diagnostic purposes.

Experimental

Design and fabrication of LoMA-B system

A LoMA-B system for analysis of DNA methylation status was composed of a sample pre-processing module and a detection module (Fig. 1-2). First, the sample pre-processing module

for on-chip bisulfite conversion was composed of a microchamber for on-chip bisulfite conversion, a three dimensional (3D) micromixer for passive mixing, and a microchannel for desulfonation and purification by capturing the modified DNA onto the surfaces, those were connected to each other with a flow path (Fig. 2). The module was fabricated using a laser cutting machine (Universal Laser Systems; Scottsdale, AZ, USA) (Fig. 2). Geometries of microfluidic networks on the module were patterned on the double-sided tape sheets (3M 9475LE; St. Paul, MN, USA) with 114 μm thickness. In order to adjust flow rate within the microchambers for the completion of DNA bisulfite conversion process, 500 μm thick PMMA was sandwiched between two patterned double-sided tape layers. Through holes were also drilled on the top layer by the laser cutting process to obtain inlet and outlet ports. Commercially available microscope glass slide (75mm x 26mm) was cleaned in piranha solution for 30min. Afterwards, protective layers on both sides of double sided tape were peeled off and the PMMA layer was placed between the cleaned glass slide (bottom layer) and top layer, and pressed gently to form microfluidic network. Connectors that consist of a hole through the center and use double-sided adhesive to connect to the microfluidic device were fabricated with 2.5 mm thick PMMA sheet. Then connectors were attached on top layers and tygon tubings (AAC02548; Cole-Parmer; Vernon Hills, IL, USA) were inserted into connectors and sealed with epoxy. Finally, Peltier thermoelectric cooler was incorporated underneath the microchamber region that needs temperature control for bisulfite conversion reaction to occur. A thermoelectric cooler controller (Series 800; Alpha Omega Instruments Corp.; Lincoln, RI, USA) was used for temperature feedback control in real time manner.

Particularly, in order to reduce complexity of the microfluidic system by eliminating the need for active components such as microvalves and micropumps from the microfluidic device, fluid flow was manipulated in continuous manner. In addition, 3D micromixer was designed to achieve the efficient mixing that can be realized by simple laser cutting fabrication without requiring any mechanical transducer within the microfluidic device. The micromixer consists of series of 3D serpentine structure in two layers, and it performs mixing by combining chaotic advection and lamination (Fig. 2c). Two fluids are primarily mixed via multi-lamination within this 3D serpentine structure as the flows are split and recombined. Chaotic advection as well as lamination occurs due to this 3D serpentine structure. In order to generate transverse flow,

existing 3D split-and-recombine (SAR) structure is modified to introduce expansion effect. To evaluate the mixing efficiency of designed 3D serpentine micromixer, computational fluid dynamics (CFD) simulations under the steady-state condition were conducted. Simulation works were conducted using CFD software COMSOL Multiphysics. In simulation, we numerically evaluated mixing performance within the micromixer composed of four mixing units. Fluid flows were introduced into mixer through two inlets and exits through one outlet. One stream carried the tracer. Dimensions of channels are the same as fabricated device. The total length of domain in simulation was ~20 mm. The diffusion coefficient of a solute (species) in water was set to 1 x 10^{-10} m^2/s. For discretization, second order elements were used for velocity and first order elements for pressure. The total number of domain elements, boundary elements, and edge elements were 32040, 24344 and 4817 respectively. The number of degrees of freedom was 973014. The total pressure drop across mixer was calculated and the mixing result was visualized (Fig. 2c).

Work flow of LoMA-B system

The work flow of LoMA-B system for the analysis of DNA status is illustrated in Figure. 1. The sample pre-processing module is used for on-chip DNA bisulfite conversion. To prepare the bisulfite solution in the sample pre-processing module, 0.832 g of sodium bisulfite ($NaHSO_3$) was dissolved in 1 ml of 0.7 M sodium hydroxide (NaOH) solution, and incubated at 50°C for 10 min. The final solution was carefully monitored, and adjusted to ~pH 5.4. Sodium bisulfite was purchased from Sigma-Aldrich. Meanwhile, 10 mg of hydroquinone was dissolved in 2.5 ml of dH_2O. Next, 1 ml of bisulfite solution and 10 µL of hydroquinone solution was mixed by vortexing. Before the bisulfite treatment, genomic DNA (gDNA) was incubated in 10 µL of 0.3 M NaOH at 37°C for 10 min to denature the double stranded DNA. Then the DNA denatured was mixed with 110 µL of mixture of the bisulfite solution and hydroquinone. Before loading the mixed sample into the sample pre-processing module, the module was filled with distilled water (dH_2O) for pre-wetting the inner surfaces, and for preventing evaporation during the reaction. Then the mixture of DNA and the bisulfite solution was loaded into the module through Inlet 1 at a flow rate of 10 µL/min using a syringe pump. The microchamber was heated, and kept at 70 °C by using a Peltier heater, which is attached underneath of the bottom of the microchamber. After

the solution went through the bisulfite treatment, it was mixed with 7 M guanidine hydrochloride (GuHCl) (Inlet 2) in 3D micromixers at a fluid velocity of 6.4 mm/s. Then the modified DNAs were adsorbed onto microchannel surface by electrostatic interaction. Washing buffer (90 % ethanol) was added through Inlet 3 for 5 min at 10 µl/min to remove residual bisulfite salt. After the washing step, desulfonation buffer (900 µL of 100 % ethanol was mixed with 100 µL of 3M NaOH) was injected, and sit for 10 min at room temperature. Finally, 90 % of ethanol was injected to wash for 5 min. TE buffer (pH 8.0) was used to get rid of ethanol for 2 min, and then it was used for DNA elution for 5 min. The eluted DNA was analyzed by using a detection module of the LoMA-B system.

For the detection module, the modified protocol was used for the detailed fabrication and structure of silicon microring resonator (SMR) sensor used for DNA amplification and detection that has been previously described.[44] For the analysis of DNA methylation status using ISAD as the detection module, either methyl-specific (M primer set) or unmethyl-specific (U primer set) primer sets were immobilized onto the sensor surface. Functionalization of the sensor surface was performed according to the protocol described previously.[40,45] Briefly, to immobilize forward primer onto sensor chip surface, 100 µl of 1 µM RARβ gene primer solution containing 20 mM sodium cyanoborohydride was added to the sensing area, and incubated for 16 h at room temperature. After the incubation, the chips were washed with MES buffer and dH_2O to remove any unbound primers and residual buffer, and then dried it with N_2 gas. Finally, PMMA well (6mm x 1.5mm x 1mm) was attached onto the chip as a sample loading well. Using ISAD as a detection module, the modified protocol used as previously described.[40,45] Finally the detection module was placed on top of heater to start amplification reaction at 37 °C, and the resonance wavelength spectrum was collected every 5 min up to 30 min in wavelength range of 1530-1580 nm.

For the reproducibility and reusability of the LoMA-B system, we examined the long-term stability of the detection module and reusability of the sample pre-processing module as followed protocol. First, the primers were immobilized on functionalized surface of SMR chips as illustrated in Fig. 1. After the immobilization process, the chips were vacuum-sealed in foil pouches, and stored at 4°C for 0, 7 and 14 days, respectively. Then the chips were used for detection of DNA methylation of input genomic DNA (1 µg) by measuring resonant wavelength

shift after 30 min amplification as described above.

Conventional bisulfite conversion with MS-PCR and RT-PCR

For extraction of human genomic DNA, an epithelial cell line (MCF-7) derived from human mammary gland cells were maintained in plastic culture dishes with high-glucose Dulbecco's modified Eagle's Medium (DMEM, Life Technology) supplemented with 10% fetal calf serum (FCS) in a 37 °C humid incubator with 5% ambient CO_2 as previously described.[45] Genomic DNA was extracted from the cell line in the presence of proteinase K using a QIAmp DNA mini kit (Qiagen; Hilden, Germany). Bisulfite conversion in solution as a conventional method was performed using CpGenome DNA modification kit (Millipore-Merck) to compare with the current system. Conventional MS-PCR and RT-PCR carried out as reference methods in order to verify DNA methylation status after the conversion. All primer sets used are described in Table S1. Both methods were used the protocol that has been previously reported.[24,45]

Results and Discussion

Working principle of a LoMA-B system

The working principle of the LoMA-B, including a sample pre-processing module and a detection module, is illustrated in Fig. 1. To discriminate between the methylated and unmethylated alleles using the LoMA-B system, on-chip bisulfite conversion was performed as a pretreatment step in the microfluidic device (Fig. 1A, Sample pre-processing module) followed by ISAD as a rapid detection step (Fig. 1B, Detection module). The LoMA-B analyzed the DNA methylation status within 80 min. First, in the sample pre-processing module (Fig. 1A), genomic DNA (gDNA) extracted from MCF-7 cells was mixed with sodium bisulfite solution in order to convert unmethylated cytosines to uracil at 70°C for 20 min (Fig. 1A_i). After conversion in the microchamber, the modified gDNA was mixed with the chaotropic agent by passing through the 3D micromixer, and then the mixed solution flowed into another microchannel and was captured on a glass surface at room temperature (RT) for 5 min. Then, the microchannel was washed with ethanol for 5 min. For the desulfonation step (Fig. 1A_ii), NaOH solution was injected into the microchannel at RT for 10 min. Finally, the modified DNA was eluted with TE buffer. After the single-chip bisulfite conversion in the sample pre-processing module (A), the modified DNA was

amplified in the detection module (Fig. 1B) with methyl-specific primer grafted onto the silicon microring resonator (SMR) at 37°C for 10–30 min in a real-time and label-free manner for the ISAD reaction. In the detection module (Fig. 1B), when the original sequences contained methylated cytosine, the resonant wavelength shifted due to the change in refractive index cause by the amplification of the modified template with methyl-specific primer. Similarly, the resonant wavelength shifted upon the amplification of the modified template with unmethyl-specific primer when the original sequences contained unmethylated cytosine. Taken together, the results demonstrate that the LoMA-B system can analyze the status of DNA methylation within 60–80 min including sample pre-processing (50 min) and detection (10–30 min).

In order to avoid inefficient sample transfer during each processing step, a continuous fluid flow system based on a single microfluidic channel was devised using multi-layered fabrication (Fig. 2). After bisulfite conversion in the microchamber, a three-dimensional (3D) serpentine micromixer was used to enhance the mixing efficiency of modified DNA with a chaotropic agent. The width of the 3D micromixer channel was 400 μm in order to reduce a drop in pressure. A schematic of the microfluidic device is shown in Fig. 2C (Left). The inset provides the detailed structure of the 3D micromixer. The height of the micromixer is 114 μm and the length of one mixing unit is 500 μm. To evaluate the mixing efficiency of the designed 3D micromixer and identify the required minimum mixing length numerically, a computational fluid dynamics (CFD) simulation under the steady-state condition was carried out using *COMSOL Multiphysics*.[46] In order to calculate the mixing results, the Navier-Stokes and convection-diffusion equations were utilized. The mixing result is shown in Fig. 2C (Right). The results clearly show that sufficient mixing of the two fluids can be achieved within four units.

Characterization of the sample pre-processing module by using MS-PCR

Next, in order to characterize the sample pre-processing module alone as an on-chip bisulfite conversion method (Fig. 1A), we used human whole genomic DNA extracted from MCF-7 cells for the bisulfite conversion of the nucleotide sequence, followed by MS-PCR (Fig. 3). To identify the optimal solution for reducing incubation and hands-on time during bisulfite conversion,[47] we first compared efficiency when using either high molarity/high temperature (High MT, 8 M/70°C) or low molarity/low temperature (Low MT, 4.9 M/55°C) conditions. The

bisulfite conversion in the High MT condition was achieved about 100% and the reaction time ranged from 40 min to 2 h, but the reaction time for the Low MT condition was 2 h (Fig. 3A). In addition, a range of reaction times from 5 min to 4 h in the High MT condition was examined to determine the minimum reaction time required using the sample pre-processing module (Fig. 3B). After bisulfite conversion, desulfonation, and purification was performed in the module, the modified DNA was eluted with TE buffer and analyzed by MS-PCR (Fig. 3A–B). The methylated *RARβ* gene was successfully amplified with methyl (M)-specific primer after a 20 min reaction and showed no amplicon with the wildtype (W) primer, demonstrating that high bisulfite conversion was achieved under the high MT condition. Therefore, we showed that the sample pre-processing module could achieve a high gDNA bisulfite conversion in the optimized conditions (50 min at High MT). To verify the utility of the sample pre-processing module as an on-chip bisulfite conversion platform, we compared the bisulfite conversion status of the current module with the conventional bisulfite conversion method (Fig. 3C–D and Fig. S1). Fig. 3C shows that the methylated *RARβ* gene was amplified with M primer after the modification reaction using the current module for 50 min, but no amplicon is observed when the W primer was used (Fig. S1B). Although the amplification of the *RARβ* gene using the conventional method for over 16 h was similar to that of the current module, the overall reaction and hands-on time including the conversion, desulfonation, and purification steps was greatly reduced using the current module (Fig. 3). Furthermore, the current module can be used to convert as little as 100 ng of input DNA within 50 min as shown in Fig. 3D and Fig. S1B. Therefore, the sample pre-processing module alone could be useful as an on-chip bisulfite modification platform to reduce both the hands-on and processing time and to avoid the inefficiency of sample transfer.

Analysis of DNA methylation status using the LoMA-B system

Next, to characterize the LoMA-B system including the sample pre-processing module and detection module for rapid and simple analysis of DNA methylation status, we combined a label-free and real-time-based ISAD technique as a detection module after the modification reaction by the sample pre-processing module. We compared the simple and rapid LoMA-B system with conventional methods including MS-PCR and real-time (RT)-PCR after the modification reaction of the sample pre-processing module alone (Fig. 4A). We showed that the resonant

wavelength shift was above 660 pm after 20 min when the methylated *RARβ* gene was amplified with the M primer (Fig. 4B). However, when the methylated *RARβ* gene was not amplified with unmethylation (U)-specific primer, the wavelength shift was only around 350 pm, which was background signal caused by non-specific targeting, as discussed previously.[30] Therefore, the LoMA-B system can be used to clearly discriminate between methylated and unmethylated alleles after the on-chip bisulfite conversion with rapidity (within 60–70 min) and simplicity (Fig. 4B). After undergoing sample pre-processing, the modified DNA was also amplified with the M primer within 3 h using both MS-PCR (Fig. 4C_rectangle) and RT-PCR (Fig. 4C). No PCR amplicons were observed when the U and W primers were incorporated with the modified DNA (Fig. 4C). The results from the conventional methods also showed the difference between the M, U, and W primers in the presence of the modified DNA (Fig. 4C). Additionally, in order to verify whether the M primer was hybridized with the methylated DNA only, we used unmodified DNA (no bisulfite conversion of gDNA from MCF-7) as a control target for hybridization with the W primer only. As a result, the M primer was hybridized with the methylated *RARβ* gene and not with the unmethylated *RARβ* gene (data not shown). Furthermore, we investigated the long-term stability and reusability of the LoMA-B system (sample pre-processing module and detection module). Therefore, we observed that the LoMA-B can be stored for at least 2 weeks and used up to 3 times for the detection of the DNA methylation (Fig. S2).

Sensitivity of the LoMA-B system in mixed samples

Finally, we evaluated the detection limit of the LoMA-B system with serially diluted samples of methylated DNA mixed with unmethylated DNA. Using serial dilutions, the gDNA obtained from MCF-7 and Jurkat cells were used as templates for the heterozygous methylated and unmethylated *RARβ* gene, respectively. To determine the minimal detection limits for the methylated DNA against an unmethylated DNA background, the gDNA (1 μg) was extracted from cell mixtures containing 100%, 50%, 10%, 1%, and 0% methylated DNA. Fig. 5A shows that the methylated DNA is easily detected using the LoMA-B system by measuring the differences in the wavelength shifts between the methylated and the unmethylated samples for 30 min. Using the LoMA-B system, the methylated DNA was detected within 80 min in dilutions containing as little as 1% methylated DNA (Fig. 5A–B). However, the methylated DNA

processed using RT-PCR was only able to detect methylation in samples containing >10% methylated DNA (Fig. 5C). The detection limit of the LoMA-B system (<1% methylated DNA) is superior to that of RT-PCR because of the inherent limitations of RT-PCR. For example, the use of intercalating dye results in a fluorescence signal from any double-stranded DNA, eliminating the ability to distinguish between specific and non-specific targets. Non-specific DNA amplification (i.e., primer-dimers) in RT-PCR is observed as background and requires an additional step for confirmation (melt curve analysis). Thus, the cut-off for reporting the methylation status by RT-PCR was set based on the results from both no-DNA and unmethylated DNA groups (>24 C_T). Therefore, the LoMA-B system is less hands-on, does not require long processing because of the single-channel microfluidic device, and detects methylated DNA using the ISAD technique even in a heterogenous population of cancer cells.

Conclusion and outlook

We have developed a LoMA-B system that can be used for a variety of DNA methylation related biomarker-based epigenetic testing applications in both research and diagnostic applications. To the best of our knowledge, this is the first LOC system based on bisulfite conversion developed for the analysis of DNA methylation status of human genomic DNA obtained from cancer cells. Despite the limitations of bisulfite conversion in solution which include complicated steps, long processing and handling time and low conversion efficiency, the bisulfite conversion-based technique is still the gold standard for the analysis of DNA methylation in clinical specimens. We demonstrated in this study that the LoMA-B system can successfully analyze the DNA methylation status of the *RARβ* gene within 80 min by combining on-chip bisulfite conversion with rapid DNA amplification/detection bypassing the limitations. Therefore, the LoMA-B system can be a potential method for analyzing the DNA methylation from human gDNA samples with rapidity, simplicity, and sensitivity as compared to other PCR-based methylation techniques (Table S2). Furthermore, this approach facilitates independent optimization and reconfiguration of the sample pre-processing module (on-chip bisufite conversion) and sensing module (detection). Although ISAD has been used as a detection method in this study, the sample pre-processing module can be applied to a variety of methylation detection methods including MS-PCR, MS-HRM, and MethyLight. The LoMA-B

system needs further validation to allow robust and diverse DNA methylation analysis with large clinical samples. Nevertheless, this LoMA-B system has great potential for use in the analysis of DNA methylation status, and adaptation to existing bisulfite-based sequencing techniques as a cancer diagnostic tool for both research and clinical use.

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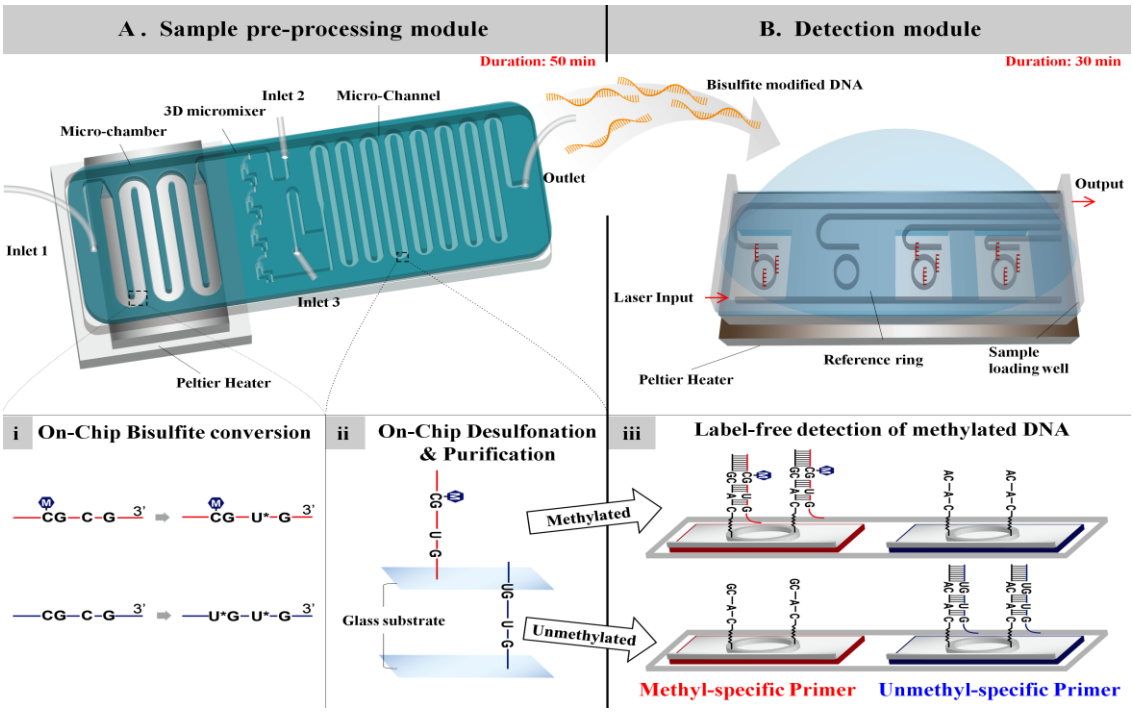

Fig. 1. Schematic of Lab-On-a-Chip system for DNA Methylation Analysis based on bisulfite conversion (LoMA-B). LoMA-B combined a sample pre-processing module and a detection module for analysis of DNA methylation. (A) A sample pre-processing module for on-chip DNA bisulfite conversion consists of a microchamber, a 3D micromixer, and a microchannel. Peltier heater is incorporated with microfluidic device to maintain the temperature for on-chip bisulfite reaction. (i) Human gDNA with bisulfite solution was loaded into the module using Inlet 1, and incubated at 70 °C for 20 min in continuous flow passing through microchamber region. (ii) Then, the bisulfite converted DNA was mixed with chaotropic buffer through 3D micromixer, and bound onto the surface of microchannel for desulfonation and purification step. Unmethylated cytosines were converted to uracils while methylated cytosines remained intact. (B) A detection module employs ISAD technique after the immobilization with either methyl or unmethyl-specific primer for the analysis of DNA methylation status. The modified DNA mixed with RPA solution was loaded onto sensing window of the SMR sensor and resonant wavelength shifts were observed during the reaction. (iii) Amplification of methylated DNA occurs on the SMR sensor functionalized with the methyl-specific primer while no amplification occurs on the chip with the unmethyl-specific primer or vice versa.

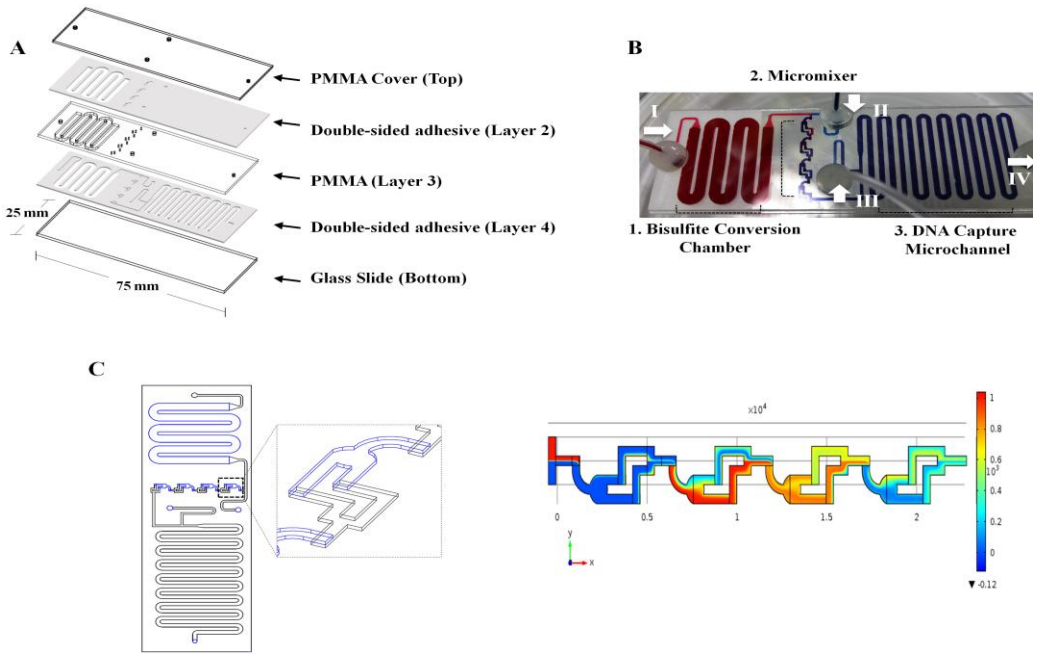

Fig. 2. Design and fabrication of a sample pre-processing module for on-chip bisulfite conversion. (A) The sample pre-processing module is fabricated with PMMA sheet by laser cutting machine for simple and rapid prototyping. (B) Photograph of the fabricated device. The microchamber is filled with red dye represents bisulfite reaction region. Blue dye represents chaotropic buffer injected from inlet II. Two fluids are mixed completely through 4 units of 3D micromixer, shown as purple in microchannel. (C) Schematic of a sample pre-processing module design (Top view) and numerical mixing simulation result by Comsol Multiphysics at a flow velocity of 6.4 mm/s in 3D micromixers (Top view).

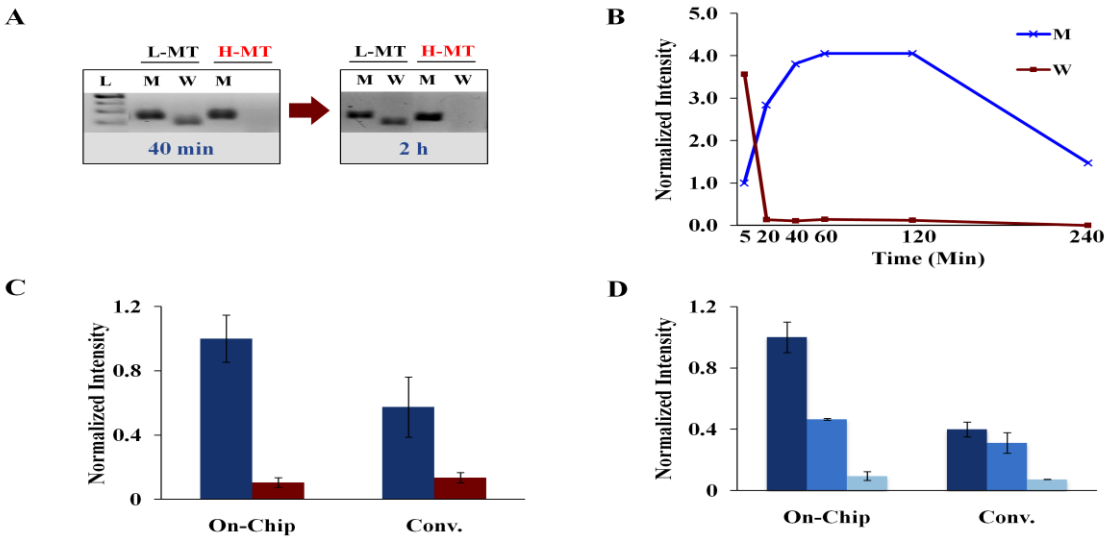

Fig. 3. Characterization of a sample pre-processing module for on-chip bisulfite conversion by MS-PCR. (A) Genomic DNA (1 μg) from MCF-7 cells was used as a target of *RARβ* gene. Gel electrophoresis of amplicons by MS-PCR using the sample pre-processing module with either Low MT (L-MT) or High MT(H-MT) condition. (M: Methyl-specific primer, W: wild-type primer, L: 50bp DNA ladder). (B) Analysis of bisulfite conversion status using the sample pre-processing module with either methyl-specific (blue) or wild-type (red) primer depending on the reaction time of the bisulfite conversion by monitoring of normalized intensity of products from MS-PCR. (C) Comparison of bisulfite conversion status of *RARβ* gene between the sample pre-processing module and the conventional off-chip bisulfite conversion method with input gDNA (1 μg) from MCF-7. The normalized intensity of products was observed using MS-PCR with methyl-specific (blue) and wild-type (red) primer. (D) Analysis of *RARβ* gene methylation pattern depending on the amounts of DNA (1 μg, 500 ng, and 100 ng) from cells. Gel electrophoresis of *RARβ* by both methods (On-chip and conventional bisulfite conversion).

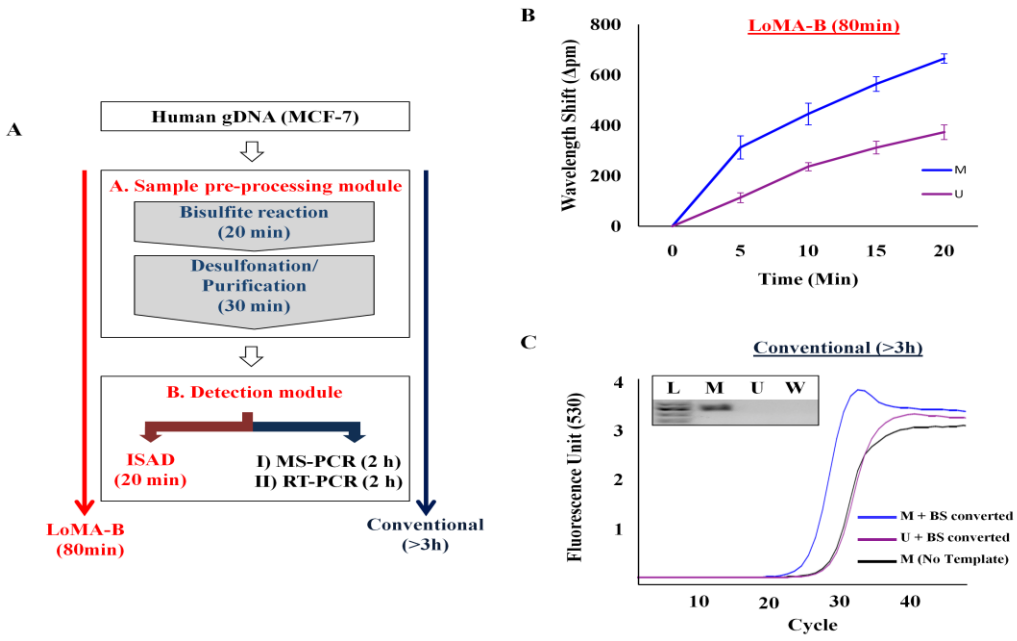

Fig. 4. Comparison of DNA methylation analysis time of LoMA-B system and conventional methods (MS-PCR and RT-PCR). (A) Work flow of analysis time using LoMA-B (80 min) and conventional methods (> 3h). (B) Analysis of DNA methylation status by measuring resonant wavelength shift during amplification on a detection module (10-20 min). The modified DNA from the sample pre-processing module (50 min) was used as a target. Methyl-specific (blue) or unmethyl-specific (purple) primers of RARβ were immobilized on the SMR for the ISAD reaction. (C) Analysis of DNA methylation status using MS-PCR (rectangle) and real-time PCR. Gel electrophoresis of amplicons by MS-PCR after the sample pre-processing module (M: Methyl-, U: unmethyl-specific primer, W: wild-type primer, and L: 50bp DNA ladder). Fluorescence signal by RT-PCR. Blue (methylated target with methyl-specific primer), purple (methylated target with unmethyl-specific primer), and black (no target with methyl-specific primer).

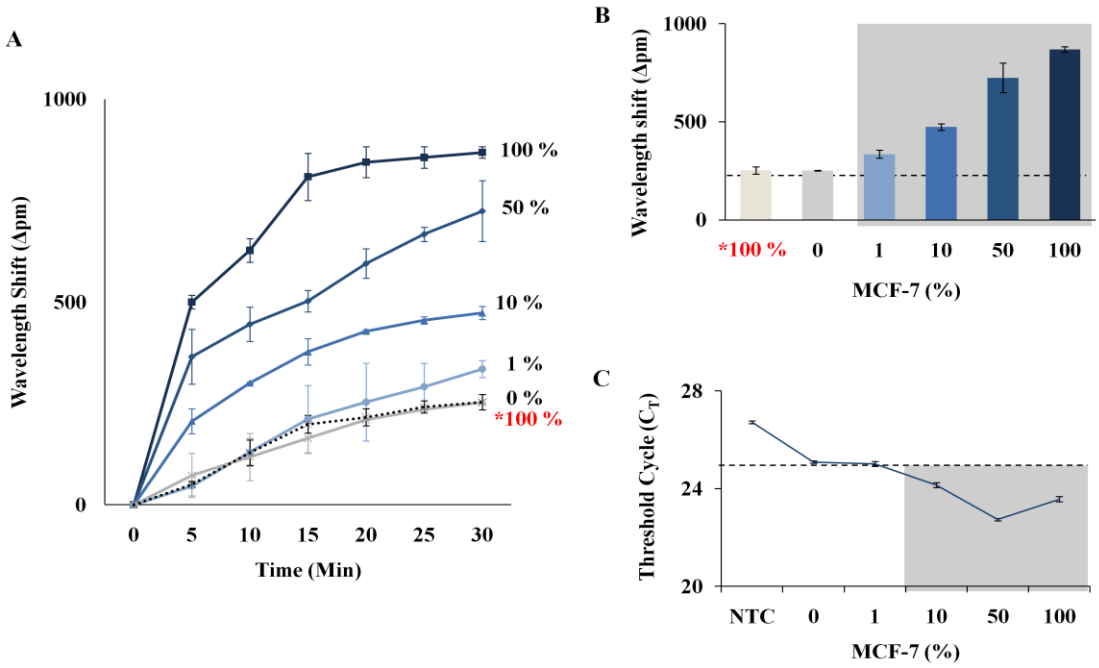

Fig. 5. Quantitative analysis of DNA methylation status with serially diluted cancer cells. MCF-7 cells containing promoter methylation of *RARβ* gene were diluted with Jurkat cells (containing unmethylated *RARβ*). The ratio of MCF-7 cells were 0%, 1%, 10%, 50% and 100%. Human gDNA (1 μg) extracted from the mixture of MCF-7 and Jurkat cells was analyzed by the LoMA-B system or RT-PCR. Error bars indicated standard error of the mean based on at least 3 independent experiments. (A) Resonance wavelength shift in LoMA-B system. The colors represent the amount of the target: Navy (100% with Methyl-specific primer), dark blue (50% with Methyl-specific primer), blue (10% with Methyl-specific primer), light blue (1% with Methyl-specific primer), grey (0% with Methyl-specific primer), and dotted-line (*100% in red with unmethyl-specific primer). (B) Resonance wavelength shift in 30 min. The methylation status of *RARβ* gene was successfully analyzed from the sample containing MCF-7 as little as 1 %. Dashed line indicates the cut-off value for the detection of the methylated DNA determined by the resonant wavelength shift of samples from MCF-7 (0%) and *100 % (100% with unmethyl-specific primer). (C) Analysis of DNA methylation status with the diluted samples using RT-PCR. The DNA methylation of *RARβ* gene was detected from the sample containing MCF-7 more than 10 %. Dashed line indicates the cut-off value for the detection of the converted DNA determined by threshold cycle value of samples from MCF-7 (0 %) and no DNA sample (NTC).

LoMA-B: A rapid and simple lab on a chip system based on single-channel bisulfite conversion for DNA methylation analysis

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Abstract

Here, we present a Lab-on-a-Chip system for DNA Methylation Analysis based on Bisulfite conversion (LoMA-B), which is coupled to a sample pre-processing module for on-chip bisulfite conversion and a label-free, real-time detection module for rapid analysis of DNA methylation status using an isothermal DNA amplification/detection technique. The LoMA-B system is an efficient diagnostic tool for the simple, fast, and quantitative evaluation of DNA methylation patterns for clinical applications.