

# A DNA methylation assay for detection of ovarian cancer cells using a *HpaII/MspI* digestion-based PCR assay in an integrated microfluidic system

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**Abstract** Early and accurate diagnosis of cancer plays a very important role in favorable clinical outcomes. DNA methylation of tumor suppressor genes has been recognized as a diagnostic biomarker for early carcinogenesis. The presence of 5-methylcytosine in the CpG islands in the promoter region of a tumor suppressor gene is an important indicator of DNA methylation. However, the standard detection assay utilizing a bisulfite treatment and *HpaII/MspI* endonuclease digestion is a tedious and lengthy process and requires a relatively large amount of DNA for testing. In this study, the methylated DNAs of various tumor suppressor genes, HAAO, HOXA9 and SFRP5, were chosen as candidates for detection of ovarian cancer cells. The entire experimental process for the DNA methylation assay, including target DNA isolation, *HpaII/MspI* endonuclease digestion, and nucleic acid amplification has been realized in an integrated microfluidic system. The limit of detection using this developed system has been experimentally determined to be  $10^2$  cells/reaction. The entire process from sample loading to analysis of the results only took 3 h which is much faster than the existing protocols. Different sources of biosamples, such as cells, ascites and serums, could be detected with the methylated DNA,

indicating that this developed microfluidic system could be adapted for clinical use. Thus, this developed microsystem may be a promising platform for the rapid and early diagnosis of cancers.

**Keywords** DNA methylation · Microfluidics · MEMS · PCR · Enzyme digestion

## Abbreviations

CA-125	Cancer antigen 125
ddH <sub>2</sub> O	Double distilled water
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EMV	Electromagnetic valve
FBS	Fetal bovine serum
HAAO	3-Hydroxyanthranilate-3,4-dioxygenase
HOXA9	Homeobox A9
LOD	Limit of detection
MEMS	Micro-electro-mechanical system
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDMS	Polydimethyl siloxane
SFRP5	Secreted frizzled-related protein 5

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## 1 Introduction

In most developed countries, such as the USA, 25 % of the total causes of death annually are cancer related, while about 0.5 % of the total population is diagnosed with cancer each year (Jemal et al. 2009). The incidence of

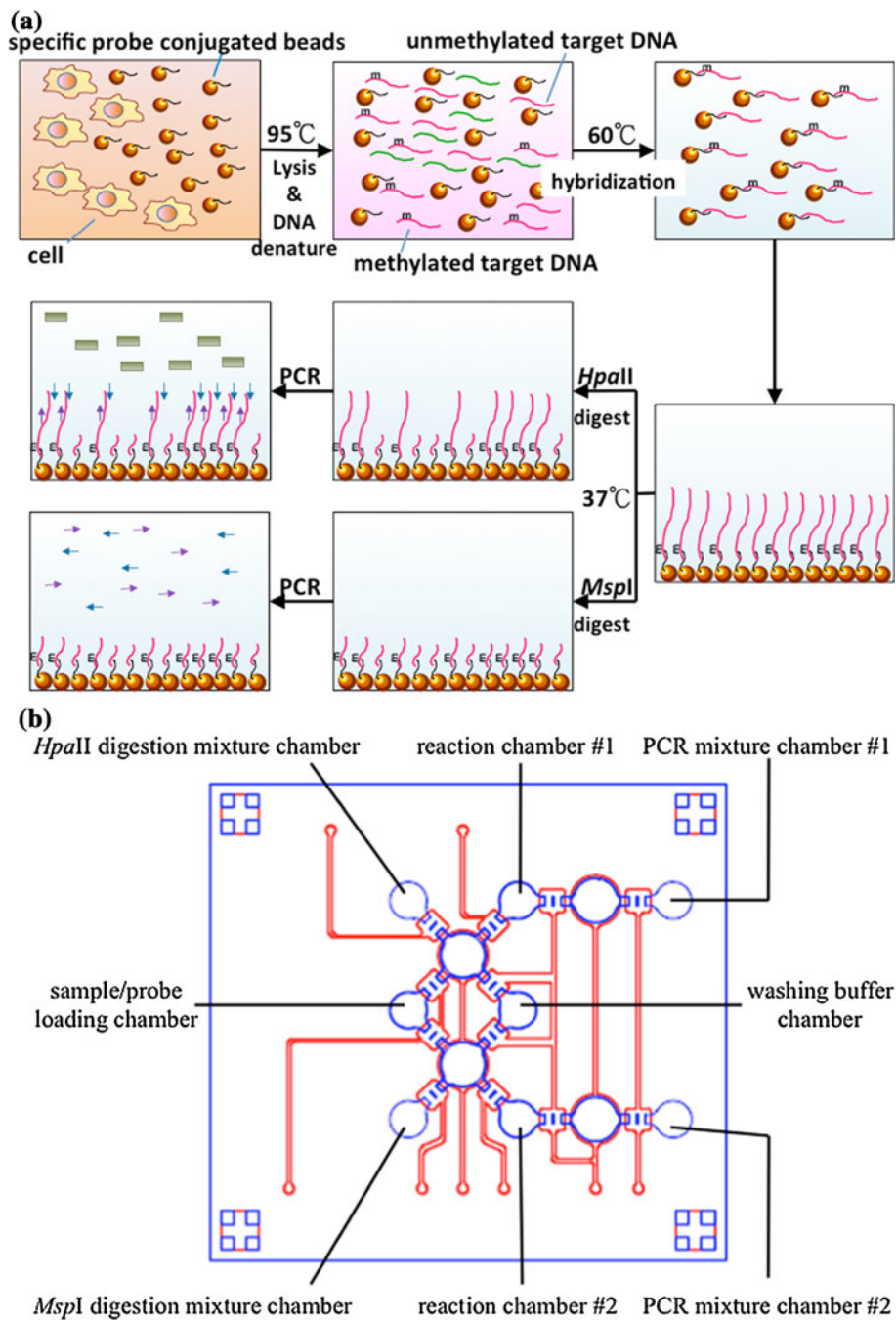
ovarian cancer was reported to be lower than 6 per 100,000 people in Taiwan. However, it had the highest mortality rate among gynecologic cancers (Annual report Department of Health 2001). The survival rate of patients usually depends on the stage of progression of the ovarian cancer. After the cancer is diagnosed, surgery combined with chemical and radiation therapies is commonly used to improve the survival rate. In traditional clinical diagnosis, the confirmation of ovarian cancer is usually performed by a physical examination, blood test for CA-125, ultrasound, in vitro diagnosis of the abdominal fluid and other biomarker indicators. The 5-year survival rate of patients with ovarian cancer that was detected at an early stage was reported to be higher than 90 % (Jemal et al. 2009). However, ovarian cancer is mostly asymptomatic or has non-specific symptoms for women in the early stages. It is therefore difficult to suspect that testing is required to diagnose for early stage ovarian cancer.

Sensitive and specific biomarkers capable of detecting early stage ovarian cancer would greatly increase the patient survival rate. Multiple genetic and epigenetic changes have been reported as important molecular indicators of ovarian cancer. These changes drive alterations in cellular signal pathways that contribute to ovarian tumorigenesis (Bast et al. 2009). More than 7 signaling pathways, 15 oncogenes and 16 tumor suppressor genes have been demonstrated to be associated with the molecular mechanisms involved in the progression of ovarian cancer (Asadollahi et al. 2010). Among them, an epigenetic alteration was demonstrated as one of the biomarkers in tumors. Epigenetic alteration is defined as a stable alteration in gene expression without any change in the DNA sequence. The most recently discovered epigenetic phenomenon were DNA methylation and histone acetylation (Ellis et al. 2009). DNA methylation is a covalent chemical modification that adds a methyl ( $-\text{CH}_3$ ) group at the carbon 5 position of the cytosine ring. The modification events commonly occur in the 5'-CG-3' sequence (CpG dinucleotide). The clusters of CpG dinucleotide in the smaller DNA region, ranging from 0.5 to 5 kb, are usually called the CpG islands. Approximately half of the genes in a human being have CpG islands (Antequera and Bird 1993). Therefore, hypermethylation in the promoter region of tumor suppressor genes is an important key that inhibits the transcription to reduce gene expression (Jones 1999). DNA methylation includes global hypomethylation of heterochromatin and local CpG island methylation which commonly occurs in ovarian cancer (Widschwendter et al. 2004). The mechanism of epigenetic alteration has been explored using molecular biology techniques, including a polymerase chain reaction (PCR)-pattern assay after restriction endonuclease digestion (Sadri and Hornsby 1996), sodium bisulfite conversion (Herman et al. 1996),

immunoprecipitation (Weber et al. 2005), and mass spectrometry based methods (Ehrich et al. 2005). These methods have been routinely used for distinguishing the methylated DNA, 5-methylcytosine ( $5^{\text{m}}\text{C}$ ), from unmethylated cytosine (C). For instance, treatment of the tested DNA with sodium bisulfite may lead to the conversion of unmethylated cytosine (C) to thymine (T), while the methylated cytosine remains unchanged (Clark et al. 1994). Therefore, a methylation-specific polymerase chain reaction (MS-PCR) or sequencing using highly specific primers can be adopted to analyze DNA methylation after a bisulfite treatment. Using of bisulfite-converted DNA for DNA methylation analysis has surpassed almost all other methodologies for DNA methylation analysis, thereby becoming the gold standard for detecting changes in DNA methylation. However, the potentially incomplete conversion of the DNA and the chemical treatment may damage the DNA which will affect the PCR results. Furthermore, the bisulfite treatment is a relatively lengthy (over 16 h long) and complicated experimental protocol. The DNA methylation profile of a tumor cell was reflected in its somatic lineage, environmental exposure, genetic predisposition, and cell-type specific chromatin structure. Therefore, the recent technologies focus on quantification of the DNA methylation status of thousands of loci at the predicted DNA region, providing information to investigate the epigenetic signature of a cell (Houshdaran et al. 2010). Furthermore, the pools of methylated sites are still under investigation and therefore the accurate related location has not yet been fully understood.

To avoid the issue that the methylated sites may be located on the designed primer regions such that they may affect the PCR results for DNA methylation screening, in this study, the primers were designed to be located on the outside region of promoter region for tested tumor suppressor genes. Alternatively, two restriction endonucleases, *HpaII* and *MspI* have been commonly used in non-bisulfite treatment methods that have been developed into DNA methylation assays (Suzuki and Grealley 2010). *HpaII* and *MspI* belong to isoschizomers that recognize the same CCGG sequence in DNA. Although both enzymes could recognize the same restriction sites, *MspI* cleaves at the sequence site of  $\text{C}^{\text{5m}}\text{CGG}$ , while *HpaII* does not. If the promoter region has been methylated, the PCR product from the promoter region is amplified in the *HpaII* digested case where the region remains intact, while in the *MspI* digested case, it does not. With this approach, DNA methylation can be identified by digestion using the two restriction enzymes and a subsequent PCR amplification. Initially, the use of restriction endonucleases assays has been limited due to the relative high DNA consumption (at least 10  $\mu\text{g}$  of tested DNA). Furthermore, various non-sequenced methylated patterns in the sample DNA

**Fig. 1 a** Illustration of the working principle for detection of DNA methylation. **b** Schematic diagram of the integrated microfluidic chip. **c** An exploded view of the integrated microfluidic chip consisting of two PDMS layers and one glass substrate. A thick PDMS structure with air chambers and a thin PDMS membrane as a fluidic channel layer are used for flow control. **d** A photograph of the integrated microfluidic chip. Note that the air channel and the liquid channel are stained with red dye and blue dye, respectively. The dimensions of the chip were measured to be 6.5 cm (length) × 7.0 cm (width) × 0.5 cm (height)

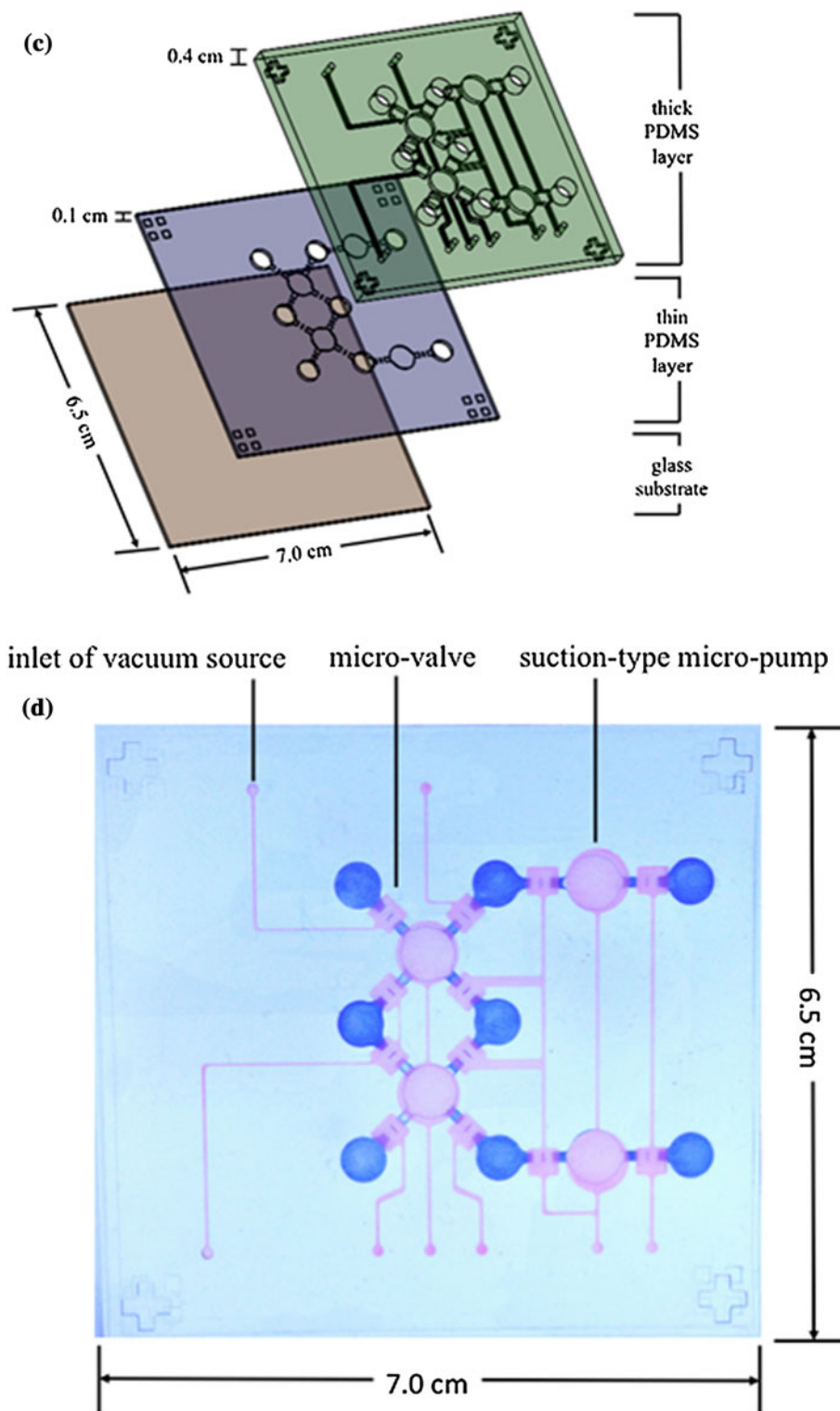


fragments could interfere within individual genomic regions (Khulan et al. 2006). However, recently, after successful sequencing of the human genome and with rapid advances in bioinformatics, the use of endonuclease digestion has been adapted for genome-wide DNA methylation analysis (Jing et al. 2012). In the developed system, although the DNA amount of  $10^3$  cells was less than 10  $\mu\text{g}$ , the specific probe-conjugated magnetic beads and MEMS

technique have more efficiency to collect the digested DNA for next PCR assay.

Recently, micro-electro-mechanical systems (MEMS) technology has been extensively used in developing compact biomedical systems since they have several advantages including low cost, disposability, low reagent and sample consumption, portability, low power consumption and the potential for automation and high-level integration when

Fig. 1 continued



compared with their large-scale counterparts. Among them, microfluidic systems have been used to study epigenetics. For instance, a chromatin immunoprecipitation (ChIP) assay has been combined with a microfluidic platform to analyze histone modification (Geng et al. 2011). The methylation of DNA in tumor suppressor promoters was

also investigated by a droplet-in-oil-type microfluidic system that performed methylation-specific PCR (MS-PCR) on a chip (Zhang et al. 2009). In addition, an opto-fluidic resonator was developed that used the MBD-2 (methyl binding) protein to capture methylated DNA (Suter et al. 2010). The isolation and purification of genomic DNA is



usually required for all the aforementioned microfluidic systems. However, there has not yet been reported a case where the module for DNA purification was integrated into the microfluidic system.

In this study, the promoter regions in some tumor suppressor genes were investigated by adopting the *HpaII/MspI* digested-PCR assay for detection of methylated DNA. The transcriptional inactivation of tumor suppressor genes is well known to be associated with DNA hypermethylation of the CpG island promoter in a cancer cell (Esteller 2008). Certain tumor suppressor genes such as HAAO, HOXA9 and SFRP5 have been demonstrated to be aberrantly methylated in cases of ovarian cancer. Several homeobox (HOX) genes are developmental genes that determine the process of differentiation for the uterus and the cervix (Du and Taylor 2004). In addition, the methylation of HOXA9 was reported to have expressed mutations in ovarian cancer tissues (Huang et al. 2009). Similarly, HAAO, known as 3-Hydroxyanthranilate-3,4-dioxygenase, is a monomeric cytosolic protein that is distributed in peripheral organs. This enzyme catalyzes the biosynthetic pathway from tryptophan to quinolinate. Some studies reported that HAAO was hypermethylated in ovarian tumors when compared with non-malignant, normal, ovarian surface epithelia using a methylation-specific PCR process (Huang et al. 2009). The epigenetic modification of the secreted frizzled-related protein (SFRP) family has been reported to play an important role in regulating Wnt signaling (Katoh and Katoh 2007). The oncogenic activation of the Wnt signaling pathway was also an indicator related to cancer progression (Su et al. 2010). Therefore, promoter hypermethylation of SFRP5 has been demonstrated to be present in ovarian cancer.

In this study, a new approach to automate a DNA methylation assay for early diagnosis of ovarian cancer cells will be demonstrated. Instead of using a sodium bisulfite treatment, which takes up to 16 h for sample pretreatment, this study attempts to shorten the entire process using endonuclease digestion. An integrated microfluidic system which combines multi-functional modules has been developed to automatically perform the entire process. When compared with other microfluidic systems reported in the literature, the entire genomic DNA from the tested cell samples could be directly extracted and purified by the specific nucleotide probe which is conjugated on the surface of magnetic beads, in the developed system. All steps of the DNA methylation assay including target DNA capture by magnetic bead-conjugated probes, *HpaII/MspI* endonuclease digestion, and on-chip PCR for amplification of tumor suppressor genes were performed on a single microfluidic system. The overall diagnosis process was more rapid with a higher sensitivity than traditional methods.

## 2 Experimental procedure

### 2.1 The processes performed by the microfluidic chip

The integrated microfluidic system used to detect DNA methylation is schematically shown in Fig. 1. With the incorporation of specific oligonucleotide-conjugated magnetic beads, all of methylated and unmethylated genomic DNA could be extracted from the tested samples (which include a BG1 ovarian cancer cell line, ascites and serums). This is followed by identifying the methylation of the samples using digestion by the restriction endonuclease *HpaII/MspI* and a subsequent PCR process. The detailed working principle is illustrated in Fig. 1a. First, ovarian cells and specific probe-conjugated magnetic beads are loaded into a sample/probe loading chamber. After thermal lysis and denaturation at 95 °C for 5 min, the genomic DNA of the tested cells are isolated by hybridizing them with the specific oligonucleotide probe-conjugated to magnetic beads at 60 °C for 10 min. Then, equal amounts of reaction complexes are transported into reaction chambers #1 and #2 by activating a suction-type micro-pump and micro-valves (Chien et al. 2009). Next, *HpaII* or *MspI* endonuclease in the digestion mixture chamber is transported into the reaction chambers and is digested with the reaction mixture at 37 °C for 15 min. Double distilled water (ddH<sub>2</sub>O) is then used as a washing buffer to remove any waste and the beads with captured DNA are collected by an external magnetic field under the reaction chamber. The PCR reagents from the PCR mixture chamber are subsequently added with the endonuclease digested complexes into the reaction chamber to perform the PCR assay. Finally, the amplified products are analyzed by off-line gel electrophoresis. All external control components such as electromagnetic valves (EMVs), vacuum pumps and waste tanks have been integrated into a developed microfluidic system for fluidic transportation (Chang et al. 2012). Note that these PCR products could be optically detected if an optical detection module and fluorescent dyes are used (Lien et al. 2009).

### 2.2 Microfluidic design and fabrication

A schematic illustration, an exploded view, and a photograph of the integrated microfluidic chip are shown in Fig. 1b–d. A microfluidic control module and a temperature control module are integrated to provide sample transportation/mixing during the diagnostic process and accurate temperature uniformity, respectively, for cell lysis, DNA hybridization, restriction enzyme digestion and PCR processes. The microfluidic control module is composed of one sample loading/bead chamber and one washing buffer

chamber. Two sets of endonuclease digestion mixture chambers, reaction chambers, PCR mixture chambers, and suction-type pumps/micro-valves are shown in Fig. 1b.

An exploded view of the integrated microfluidic chip is shown in Fig. 1c. The developed microfluidic chip is composed of several micro-devices that are integrated into the chip to automate the entire diagnosis process. It is composed of two layers of polydimethyl siloxane (PDMS) layers on a glass substrate. The top PDMS layer is used as the fluidic channel layer comprising of a sample/probe loading chamber, a washing buffer chamber, two sets of reaction chambers, enzyme digestion mixture chambers and PCR mixture chambers. The second PDMS layer is used as the air chamber layer containing the air chambers for activating the suction-type micro-pump, the micro-valves and the connecting micro-channels (Weng et al. 2011). The PDMS membrane structure is made in a ratio of 10:1 by weight of the PDMS polymer to the curing agent (Sylgard 184A/B, Sil-More Industrial Ltd., USA). The two PDMS layers and the glass substrate are bonded together to form the integrated microfluidic chip using an oxygen plasma treatment. The detailed micro-fabrication process can be found in our previous study (Lien et al. 2007). Note that, in this microfluidic system, the volume of the reaction mixture needs to be equally distributed between two reaction chambers for the analysis of the PCR results after *MspI* and *HpaII* digestion treatment. To avoid generating gas bubbles and dead volumes in the micro-pump, a suction-type micro-pump is adopted in this developed microfluidic chip (Chien et al. 2009). Fluid transport is generated by compressed air (negative pressure) that causes the deflection of the PDMS membranes in the suction-type micro-pump. Electromagnetic valves (EMVs, SMC Inc., S070M-5BG-32, Japan) and a digital controller are used to regulate the pumping rate.

Micro-heaters and a temperature sensor are used to control the temperature for the cell lysis, DNA hybridization, restriction enzyme digestion and PCR steps. The accuracy and reliability of the temperature control using the micro-heater were investigated in a previous study (Hsien et al. 2009). Briefly, the temperature can be

precisely controlled with a variation less than 0.1 °C. Note that the reciprocating actuation between the PDMS membranes of the micro-pump can be further used as a micro-mixer in this developed microfluidic chip. A photograph of this microfluidic chip is shown in Fig. 1d. The dimensions of the chip were measured to be 6.5 cm (length) × 7.0 cm (width) × 0.5 cm (height).

### 2.3 Methylated sample pre-treatment

The ovarian cancer cell line BG1, lung cancer lines A549 and H1650 were provided from the National Cheng Kung University Hospital, Tainan, Taiwan. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) (F-12 medium, Hyclone, USA) supplemented with 5 % fetal bovine serum (FBS, Hyclone, USA) and 5 % antibiotic antimycotic (Invitrogen, USA). The cells tested for DNA methylation were treated with 1× trypsin/ethylenediamine tetraacetic acid (EDTA, Biowest, France) to release them from the surface of the culture flask and were washed with a 1× phosphate-buffered saline (PBS; Biowest, France) twice. As mentioned previously, the DNA of tumor suppressor genes, HAAO, HOXA9 and SFRP5 was used in this study. Detailed descriptions of the protocols, which include the conjugation of DNA-specific nucleotide probes conjugated onto the surface of the magnetic beads can be found in our previous work (Wang et al. 2011b). The specific probe and primer pairs are listed in Table 1. They were designed using VectorNTI<sup>®</sup> software (Invitrogen, USA) and prepared as a 100-μM stock solution in ddH<sub>2</sub>O. The unbound, non-specific DNA was washed twice by ddH<sub>2</sub>O. The captured DNA on the surface of the probe-conjugated magnetic beads was collected by a permanent magnet placed underneath the chip. The captured DNA was dissolved in 5 μl of ddH<sub>2</sub>O for the subsequent experiments.

### 2.4 *HpaII/MspI* enzyme digestion-based PCR assay

For each enzyme digestion, 10 μl of reaction mixture was prepared. This mixture contained 10 U/μl *HpaII* or *MspI*

**Table 1** Designed sequences of the primer pairs for the specific DNA probe

Name	Sequence (5' → 3')
HAAO probe	C6 amide-TTTTTTTTTTGTAGCAGGAAAAGGTAGGGAG
HAAO forward primer	CGGAATTGATCTCAGGATCG
HAAO reverse primer	ACAGCCTGGAGGACAGAAAA
HOXA9 probe	C6 amide-TTTTTTTTTTTGAATACAAAGCCACGGTT
HOXA9 forward primer	CCAGTCCCCAGTTTTACAGC
HOXA9 reverse primer	TTTATTGGCATTGCCTCCTC
SFRP5 probe	C6 amide-TTTTTTTTTTGGCTTTTCTCCCTCTGCTCT
SFRP5 forward primer	CCGGCCCTGACTCTACCC
SFRP5 reverse primer	GAGAGGGGCGCAAGACCT

(New England Biolabs, Inc. MA, USA) in  $1\times$  NEB buffer 1 (10 mM Bis–Tris–Propane–HCl, 10 mM  $MgCl_2$ , 1 mM dithiothreitol, pH 7.0), and 5  $\mu$ l of bead-captured DNA was incubated at 37 °C. Then, all 15  $\mu$ l of the digested mixture was washed by ddH<sub>2</sub>O twice and collected by applying a magnetic field. The PCR mixture for the DNA methylation assay contained 1  $\mu$ l of deoxyribonucleotide triphosphate (dNTP, 10 mM, Promega, USA), 2  $\mu$ l of  $10\times$  PCR buffer (20 mM Tris–HCl, pH 8.0, 100 mM KCl, 20 mM  $MgCl_2$ ), 0.1 mM 2-[2-(Bis (carboxymethyl) amino) ethyl-(carboxymethyl) amino] acetic acid (EDTA), 1 mM Dithiothreitol (DTT), 0.5 % Tween, 0.5 % Nonidet and 50 % (v/v) glycerol (JMR Holdings, UK), 1  $\mu$ l of HAAO/HOXA9/SFRP5 specific primer pairs (0.5  $\mu$ l of each primer for the forward/reverse primers), 0.5  $\mu$ l of Superthermo Gold Taq DNA polymerase (5 U/ $\mu$ l, JMR Holdings, UK) and 10.5  $\mu$ l of ddH<sub>2</sub>O. The thermocycling process for PCR was then performed under the following conditions: 95 °C for 5 min (initial denaturation), and 30 cycles of PCR at 95 °C for 20 s (denaturing), 60 °C for 20 s (annealing) and 72 °C for 50 s (extension) for each cycle; and then finally at 72 °C for 7 min. The PCR products of 639, 1,306 and 257 base pairs (bp) were amplified for the promoter of the HAAO, HOXA9 and SFRP5 genes in this study, respectively.

### 2.5 Sensitivity and biosample testing

To determine the limit of detection (LOD) of this developed microfluidic system, a serial dilution from  $10^4$  to  $10^0$  cells of BG1, H1650 and A549 cancer cell lines were used. The methylated DNA from the tested samples in which different cell numbers and cell types were purified by specific probe-conjugated magnetic beads, digested by either the *HpaII* or *MspI* enzyme and the DNA was amplified using the protocol described previously. To determine the sensitivity of the *HpaII/MspI* digestion-based PCR assay for DNA methylation, slab-gel electrophoretic separation was used. In addition, the tested biosamples were from different sources such as ascites or serums which are typical sources for DNA methylation diagnosis in clinical laboratories. In this study, the tested ovarian cancer cell line BG1 and ascites were obtained from National Cheng Kung University Hospital, Tainan, Taiwan. The tested serums were donated from two healthy volunteers. One sample is from a male and the other is from a female where no ovarian cancer was diagnosed after a health examination. Methylated HeLa cellular genomic DNA was artificially added into a biosample as a positive control to confirm the entire protocol of the probe capture/enzyme digestion/PCR assay. Moreover, a diagnosis of the methylated HeLa DNA added to the volunteer serum was

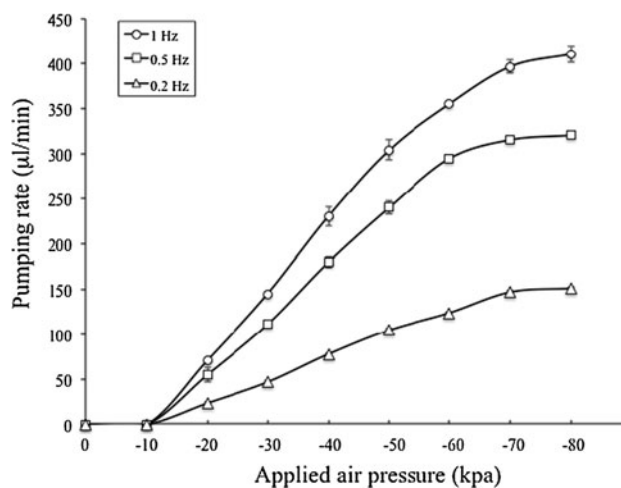
also tested. However, a maximum of 10  $\mu$ l of serum volume could be tested because the extracted serum contained an anti-coagulation inhibitor that could disrupt and cause sample clotting which would affect PCR progress at higher temperatures (e.g., 95 °C).

## 3 Results and discussion

### 3.1 Characterization of the pumping rate

The suction-type micro-pump was activated by compressed air and regulated by EMVs to control the pumping rate. This design decreased the dead volume in the transportation unit of the suction-type micro-pump (Weng et al. 2011). Figure 2 shows the relationship between the pumping rate of the micro-pump and the different applied air pressures under different EMV frequencies. The driving frequencies of 0.2, 0.5 and 1.0 Hz were tested and the pumping rate measured. The experimental results demonstrated that the pumping rate increased with an increase in the applied air pressure (suction force). A maximum pumping rate was measured to be 425  $\mu$ l/min at  $-80$  kPa when operated at 1.0 Hz.

In this study, it is important that an equal distribution of reactant be transported into reaction chambers #1 and #2 for *HpaII/MspI* digestion and PCR, which was performed by activating the micro-pump. The results from the PCR would indicate whether there was DNA methylation of a tumor suppressor gene in this study. The percent portion of the distributed reactant in each reaction chamber is calculated as follows:



**Fig. 2** Relationship between the pumping rate and the operating air pressure for the suction-type micro-pump

**Table 2** The portion of the reactant distributed between reaction chambers #1 and #2

Operating time (s)	Portion of the distributed reactant in each reaction chamber (%)	
	#1	#2
0	0	0
1	23.4 ± 0.7	19.6 ± 0.9
2 <sup>a</sup>	46.5 ± 1.2	45.0 ± 1.8

Statistical analyses were performed to determine the statistical difference using the two-tail student *t* test. No statistical significance is confirmed when  $P > 0.05$

<sup>a</sup> Indicates that the transported volume in each reaction chamber has no significant difference between reaction chambers #1 and #2 after 2 s

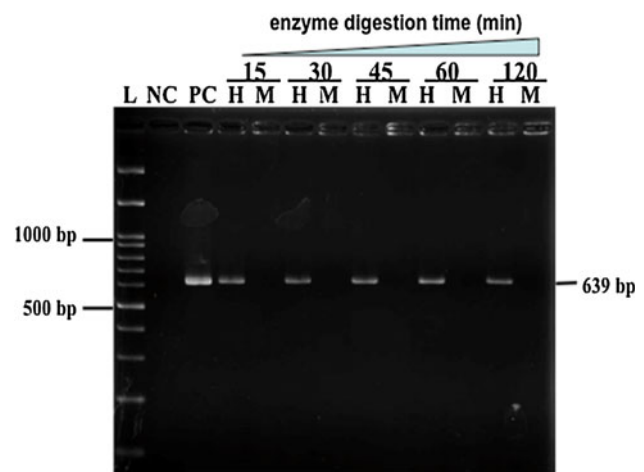
Portion of the distributed reactant

$$= \frac{\text{Transported volume in the reaction chamber}}{\text{Original volume of reactant (20 } \mu\text{l)}} \times 100 \%$$

The reactant distribution was performed at  $-80$  kPa and 1 Hz. The results of Table 2 show that the transported volume into reaction chambers #1 and #2 was not significantly different from 1 to 2 s. A statistical analysis was performed to determine if there was any statistical significance using the two-tail student *t* test. No statistical significance is confirmed when  $P > 0.05$ . The results showed the total original volume of reactant was equally distributed into reaction chambers #1 and #2 to perform the subsequent PCR processes.

### 3.2 Optimization of enzyme digestion

The optimum conditions for restriction enzyme digestion, especially the reaction time, play a critical role in the DNA methylation assay. Instead of using the sodium bisulfite treatment, which takes up to 16 h for sample pretreatment, this study attempts to shorten the entire process using endonuclease digestion. Note that the activity of the enzymes was determined by DNA digestion under different reaction times. Then the PCR results were used to determine the optimum time for *HpaII/MspI* digestion. 1  $\mu\text{g}$  of genomic DNA extracted from an ovarian cancer cell line BG1 was first captured by HAAO-specific probe-conjugated beads and purified by ddH<sub>2</sub>O washing. Then, these reactants were digested by 1 U (activity unit of the restriction enzyme) of endonuclease (*HpaII* or *MspI*) operated for 15, 30, 45, 60 and 120 min at 37 °C. Note that three reporter genes have been tested using the developed microfluidic chips. The slab-gel electropherograms shown in Fig. 3 indicate that the HAAO gene is completely cleaved by *MspI* after 15 min, whereas the *HpaII* enzyme



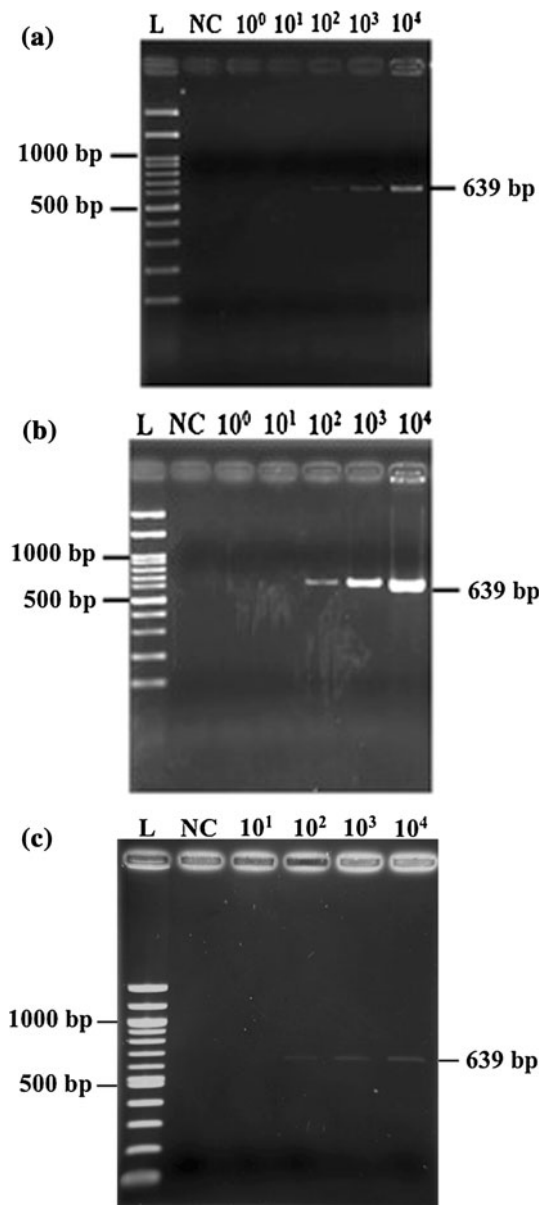
**Fig. 3** The optimization of *HpaII/MspI* digestion time for DNA methylation using the HAAO primer set in the integrated system. Lane L 100-bp DNA ladder, NC negative control using ddH<sub>2</sub>O, PC positive control using the whole genomic DNA, H the probe-captured genomic DNA digested by *HpaII*, M the probe-captured genomic DNA digested by *MspI*. Different digestion times (15, 30, 45, 60 and 120 min) were tested

still could not cleave the methylated DNA within the same period of time. In addition, the results of less than 15-min processing (e.g., 10 min) for enzyme digestion were tested. However, the result could not present high reproducibility for several repeated experiments. To assure the reliable results using the developed microfluidic system, the 15-min treatment was determined as the shortest reaction time in the developed system. The optimum digestion time for the endonuclease in this developed system is then determined to be 15 min, which is significantly a shorter time period when compared with the time required for sodium bisulfite treatment (16 h).

### 3.3 Sensitivity tests

In addition to having a rapid operating time due to enzyme digestion, improving the detection sensitivity is another important factor for developing this integrated microfluidic chip. To determine the sensitivity of the developed *HpaII/MspI* enzyme digestion-based PCR assay, the HAAO primers were designed within the promoter region of the HAAO gene using a bioinformatic assay (Wang et al. 2011a). The LOD for three cancer cell lines (BG1 ovarian cancer cell and two lung cancer cell lines, H1650 and A549) was then determined by testing serial tenfold dilutions with an initial concentration of  $10^4$  cells/reaction. The results of this sensitivity testing performed using HAAO-specific probes, endonuclease digestion and a PCR processes are shown in Fig. 4. Accordingly, the same analytic processes confirmed that all of the tested tumor suppressor genes have been tested with three cancer cell lines using





**Fig. 4** Testing the sensitivity of the DNA methylation assay performed on the integrated microfluidic chip. Different concentrations of diluted cells were detected using the HAAO primers. Three strains of cells including **a** BG1 ovarian cancer cell line, **b** H1650, and **c** A549 lung cancer cell lines were analyzed. The sensitivity is found to be  $10^2$  cells/reaction. Lane L 100-bp DNA ladder, NC negative control using ddH<sub>2</sub>O; the other lanes indicate the different cell numbers tested per reaction

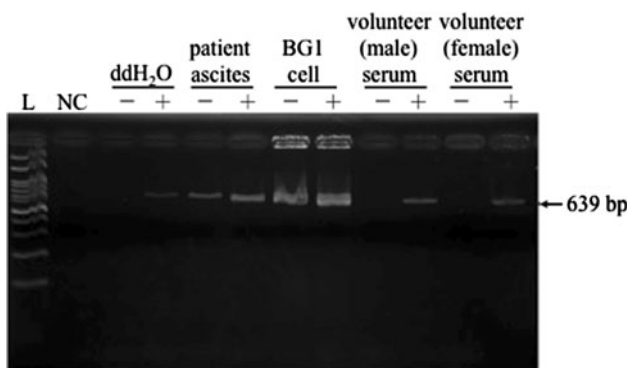
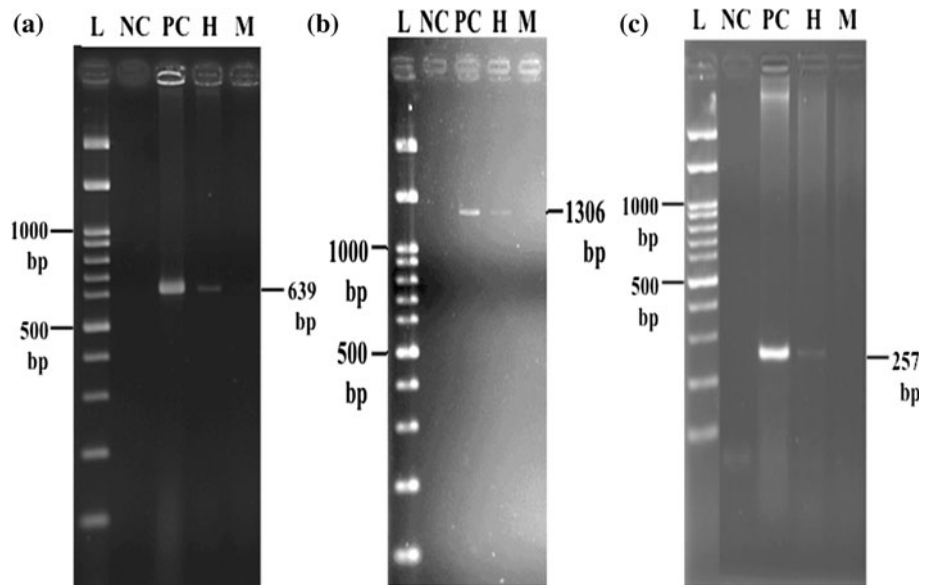
the developed microfluidic chips (data not shown). The conventional *HpaII/MspI* enzyme digestion-based PCR assay required a large amount of purified DNA (at least 10  $\mu$ g) for digestion and PCR. When compared with traditional diagnostic methods, this developed system needs a fewer number of samples. The LOD of the developed system was experimentally determined to be  $10^2$  cells/reaction for the three different cancer cell lines. Note that

each reaction required only 20  $\mu$ l. However, the specific probe-conjugated magnetic beads had a high capturing rate for DNA extraction and isolation (Wang et al. 2011b). The previous studies showed that  $2 \times 10^3$  mammalian cells were required for DNA methylation assay when using the *HpaII/MspI* digested/PCR assay (Diala et al. 1983). Therefore, our results show that this developed system for DNA methylation detection is more sensitive than conventional clinical diagnostics.

The accuracy of DNA methylation has been verified and is shown in Fig. 5. The genomic DNA of an ovarian cancer cell line BG1 was purified from  $10^3$  cells using specific probe-conjugated beads by thermolysis under 95  $^{\circ}$ C for 5 min. Both the *HpaII* and *MspI* enzymes were used to digest target DNA and the cleaved DNA fragment was washed away by ddH<sub>2</sub>O. Immediately, the degree of DNA methylation for various tumor suppressor genes in ovarian cancer cells was demonstrated by amplifying specific PCR products which were detected using slab-gel electropherograms. The PCR results showed that the methylated DNA in HAAO (639 bp), HOXA9 (1,306 bp) and SFRP5 (257 bp) could not be digested by *HpaII* and can, therefore, be amplified by specific-subtype primers/probes. However, the methylated DNA in HAAO, HOXA9 and SFRP5 gene were digested by *MspI* such that no amplified products were observed. The experimental results demonstrated that the developed system has a great potential for DNA methylation diagnosis. All of methylated DNA from the three tumor suppressor genes was accurately amplified using the *HpaII/MspI* enzyme digestion-based PCR assay.

To further verify whether this developed system could be suitable for different sources of clinical samples, methylated HeLa genomic DNA was also used as a methylation-positive control. The methylated DNA was then added into different sources (for e.g., buffer solution, ascites, cancer cell lines and serums) to test the DNA methylation assay. As shown in Fig. 6, all of the clinical samples (20  $\mu$ l) were spiked with 1  $\mu$ g of methylated HeLa genomic DNA to amplify the HAAO gene. It should be noted that the HAAO gene was also amplified from patient ascites samples ( $10^4$  cells/reaction) and the ovarian cancer cell line ( $10^4$  cells/reaction) even though no methylated HeLa genomic DNA was added. This indicates that the developed system can be used for diagnosis of cancer cells. The spiked methylated DNA in the two volunteers' serums was detectable using the *HpaII/MspI* enzyme digestion-based PCR assay. The slab-gel electropherograms showed a higher intensity of the DNA band for the cases of samples spiked with methylated DNA. The results indicate that this developed specific-probe capturing/endonuclease digestion/PCR assay could be suitable for various clinical samples and the endogenous methylated DNA may be detected from various types of clinical samples. In addition

**Fig. 5** Three types of primer sets including **a** HAAO, **b** HOXA9 and **c** SFRP5 were tested with BG1 ovarian cancer cells using the *HpaII/MspI* enzyme digestion-based PCR assay. Lane L 100-bp DNA ladder, NC negative control using ddH<sub>2</sub>O, PC positive control using the whole genomic DNA, H the probe-captured genomic DNA digested by *HpaII*, M the probe-captured genomic DNA digested by *MspI*



**Fig. 6** Testing of the DNA methylation assays with a biosample in the microfluidic chip. Different tested biosamples were detected with the HAAO3 primers. The five tested samples include ddH<sub>2</sub>O, patient ascites, BG1 ovarian cancer cell lines, serums donated from a male volunteer and from a female volunteer. Lane L 100-bp DNA ladder, NC negative control using ddH<sub>2</sub>O with the PCR reagent; the other lanes were treated with specific probe beads/restriction endonuclease digestion/PCR. The “minus” sign indicates that no methylated HeLa genomic DNA was added and the “plus” sign is for when methylated DNA was artificially added

to HAAO, the other tested tumor suppressor genes (HAAO, HOXA9 and SFRP5) have been tested and confirmed by the developed microfluidic chips (data not shown).

Note that the entire process from sample loading to observing results only took 3 h. Bio-COBRA, a modified protocol for Combined Bisulfite Restriction Analysis, was popularly used to quantify DNA methylation with an electrophoresis step in a microfluidic chip (Brena et al. 2006). The Bio-COBRA assay, when used with the Agilent 2100 Bioanalyzer platform, could detect in less than 1 h. However, if the protocol started from the DNA extraction and purification step, the entire protocol would require 48 h (Brena et al. 2006). The LOD of our developed integrated

microfluidic system is comparable with the Bio-COBRA microfluidic system, while the entire process including sample pre-treatment can be performed automatically within a shorter period of time.

#### 4 Conclusions

DNA methylation is recognized as an important biomarker for carcinogenesis. The conventional sodium bisulfite treatment required a lengthy process before detection of DNA methylation, even when performed on a microfluidic system. Alternatively, the *HpaII/MspI* enzyme digestion-based PCR assay could reduce the operation time. However, it still requires a large amount of DNA for the entire experimental protocol. In this study, specific probe-conjugated magnetic beads were used to capture methylated DNA in the test samples. The LOD of the DNA methylation assay performed in this microfluidic system showed that it only took 10<sup>2</sup> cells/reaction. The entire process from sample loading to observing diagnostic results only took 3 h. Consequently, this integrated microfluidic system for detection of DNA methylation may provide a powerful platform for rapid and sensitive detection of ovarian cancer.

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