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Development of isothermal digital PCR platform for quantification of circulating tumor DNA

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최 문 석

Abstract

Development of isothermal digital PCR platform for quantification of circulating tumor DNA

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Quantification of circulating tumor DNA facilitate detection of cancer without invasive detection and variables derived from characteristic of cancer patients against conventional cancer detection technique such as anti-body bio marker or tissue biopsy. For the detection and quantification of DNA biomarker, gene relate technologies are developed actively employing DNA amplification technique based on polymerase chain reaction (PCR). Especially, digital PCR specialized on quantify assay is commonly employed

for research about the DNA samples which have extremely low concentration in entire liquid sample. But conventional digital PCR process generally require high cost equipment, well-trained technicians and time consuming caused by complex steps. So, the researches about microfluidic systems to substitute conventional digital PCR are on the progress in the contemporary society. In this study, a microfluidic platform that have possibility of quantification of ctDNA without high cost equipment and time consuming is through employing microfluidic systems and isothermal DNA amplification known as recombinase polymerase amplification (RPA). With isothermal DNA amplification technique, the limitations derived from conventional PCR, such as high temperature, are not remained. And also, designed microfluidic platform facilitate robust, fast and simple colony forming which is key-point of the digital PCR technics for the quantification of DNA samples. With this platform, liquid samples are easily isolated in 20,000 micro well structures with simple pressing force and generic DNAs extracted cancer cells are used to mimic actual ctDNA environment and extremely low concentration in patient blood samples. Extracted generic DNAs with mutation are detected with designed primers and probes successfully in microfluidic platform. This study provide the robust and easy-to-

use microfluidic platform for quantification of ctDNA as point-of-care (POC) platform.

keywords : Microfluidics, Digital PCR, High-throughput, ctDNA detection, Diagnostics
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Table of Contents

List of figures

of micro well structure. Micro wells are fully isolated locate on 400 μm from the side wall of micro fluidic channel. 92% of whole micro wells are fully isolated under full pressing force. Scale bar = 200 μm.

Figure 2.3 Schematic of isothermal digital PCR process in micro fluidic platform. In each 20,000 micro wells, reagents and generic DNA extracted from tumor cell perform isothermal DNA amplification process. End-point image after DNA amplification process appear as the below figure. From the left to middle, each color of fluorescence represent different DNA amplifications and from the right image, amplification processes in this platform progressed independently in fully isolated micro wells without the interaction of surrounding wells.

Figure 3.1 End-point fluorescence image of target DNA after isothermal DNA amplification (TP53 extracted from SW620 and MD-AMB231). (A) Bright field image of micro well area. (B) Fluorescence image of TP53 extracted from MD-AMB231 with initial template concentration = 10^8 copies/μl. (C) Fluorescence image of TP53 extracted from SW620 with initial template concentration = 10^8 copies/μl. (D) Merged image. (E) Merged image with gray scale image. Amplification of DNA is occurred in fully

isolated micro wells. Scale bar = 200 μm.

Figure 3.2 Real time fluorescence signal image and graphical figure of fluorescing micro wells on the process of DNA amplification. Each image show fluorescence signal increasing during amplification process. From beginning of amplification to 30 min after, fluorescence signal numerical value increase dramatically. With the standard of fluorescence signal value, wells with more than doubled fluorescence count as fluorescing wells after 30min.

Figure 3.3 End-point images of fluorescence detection in microfluidic platform after digital RPA process with diverse initial template concentration conditions and quantification of digital RPA with graphical figure. (A)Each image represent the result of different initial template concentration conditions after digital RPA process in microfluidic platforms. According to the increase of initial concentration of templates, the fluorescing wells in end-point increase. All the images contain 2,000 wells (10% of whole micro wells) and the wells which have mutated DNA in initial state appear with high intensity of fluorescence in the final state. During 40min amplification at 39℃, each DNAs isolated in the micro wells amplified with the increase of fluorescence intensity

without interaction between surrounding wells. (B)According to initial template concentrations, the number of fluorescing wells increased. The sections from the control (0 copies/㎕) to 10^3 copies/㎕ condition stand for extremely low concentration of ctDNA in actual cancer patient blood sample and robust detection in this sections signify the possibility of usage on the site.

Chapter 1. Introduction

1.1 Study Background

Cancer is one of the most critical diseases in entire modern society and advanced tumor therapy requires comprehension of characteristics of various tumor cells in molecular level such as colon cancer, breast cancer. [1-3]. Because tumors evolve to resist on conventional therapies and the information from barely obtained tumor tissue is restricted, additionally [4, 5]. For this reason, circulating tumor DNA (ctDNA) is considered as one of the significant bio-markers and this molecular level bio-marker is emerged from tumor cell by secretion, apoptosis and necrosis that contains various genetic information of tumor cells [6, 7]. From the information of ctDNA, point of care (POC) diagnostics and understanding of tumor cells in the nucleic acid level have the possibility to develop and form foundation stone for high-efficiency therapy of cancer [8, 9].

Point-of-care (POC) assays reflect tendency about low cost

consuming and user friendly system among high accuracy for usage on the [10]. These rapid, simple and robust assays have the possibility of quantitative assay regarding DNA/RNA detection [11]. With this characteristic of POC, quantitative assay of mutated DNA emerged from tumor cells is one of the major concept of the diagnostic researches [12]. It also requires high-throughput systems because the density of ctDNA is extremely low and when the detection processed with whole plasma sample, the signal of ctDNA detection can be considered as negligible [13-15]. Because of extremely low concentration of ctDNA, digital PCR technique is employed for detection of ctDNA commonly [9]. Digital PCR technique have kind of differences by forming colony for amplification with conventional PCR technique [16]. Through colony formation, liquid sample divided in to tens of the thousands nano, pico liter colony for quantitative assay of DNA samples and with this sample division, mutated DNA detected even with extremely low concentration in whole liquid samples with amplified fluorescence signal [17]. But Conventional high-throughput liquid biopsy quantitative assays, such as droplet fluorescence detection [18-21], microfluidic PCR chip [22-25], still require an amount of time, the cost for processing and equipment, and also technicians due to PCR process that requires

laboratory equipment [11, 26]. Also because of this high temperature demanding PCR technique, the structures and materials of the microfluidic device have been complicated and limited [27, 28].

1.2 Purpose of Research

In this paper, we will introduce the isothermal digital PCR device with simple-pressing motion derived liquid isolation in 20,000 micro wells for quantification of ctDNA with robust efficiency. This platform is fabricated with poly-(dimethylsiloxane)(PDMS) has flexibility which facilitates liquid isolation by deformation of the part of the device. With this mechanism, micro wells on the top side of the channel will be attached on the substrate and form fully isolated micro chambers. Through simple pressing motion, micro chamber array is formed in a moment without additional laboratory equipment, complex fluidic system and irreversible deformation of the channel. And in this array, the robust quantification of ctDNA in ultra-low concentration is possible with high efficiency. Additionally, Customized jig facilitate accurate compression and analysis of fluorescence detection with robust stabilization. Also, by employing one of the isothermal PCR

technique known as recombinase polymerase amplification (RPA) technique, the selection of materials and the complexity of the structure are unlimited because of statically low temperature amplification process [16, 26].

This present study aims to overcome shortcomings of conventional point-of-care technique with low cost, less time consuming and high-throughput screening. In addition, the probe system designed to detect target DNA facilitate real-time detection with fluorescence on the site without laboratory equipment.

Chapter 2. Materials and Methods

2.1 Device design and fabrication

Master mold of microfluidic digital PCR platform was fabricated by photolithography for 50㎛ height of micro post array structures on the 100㎛ height of positive relief structure on the silicon wafer using negative photo resist SU-8. The 50㎛ height of micro post array structures form hexagonal micro well arrays, shape of honey

comb structures, on the PDMS replica while 100㎛ structure form micro fluidic channel for filling of liquid sample and reagents. The 2D design of the platform was established by AutoCAD to have hexagonal micro channel with 37mm length and 10mm width. Also 20mm length with 10mm width micro well array was established on the center of micro fluidic channel. In center of the platform, 20,000 micro wells are located on the 100㎛ height wide micro fluidic channel for sample division based on the publication about particle division in micro wells [29](Figure 2.1). Dimension of micro well is optimized based on publication above and the dimension of wall thickness is optimized according to pressure force and possibility of fabrication through photolithography and soft lithography. Microfluidic platform was fabricated through soft lithography technique using PDMS (Sylgard 184, Dow Corning) from silicon wafer master mold. A prepolymer mixture of PDMS and curing agent mixed with the ratio of 10:1(w/w) was poured on the silicon wafer mold and degassed in vacuum chamber at 0.2 bar absolute pressure. After degassing, curing process proceeded on 85 ℃ hot plate for 30min and the PDMS replica is peeled off from the master mold. Inlet and outlet is formed with 1mm diameter biopsy punch on the each end of micro channel. The device is bonded with glass coverslip after treating oxygen plasma on each

surface of device and glass for 1min to make surface hydrophilic.

By applying pressure force on the center of microfluidic channel, micro well array area, the wall of micro well in PDMS part of platform is attached to the glass coverslip. For continuous pressure force applying, customized pressing jig is employed to apply force equally and fixation of the platform.

2.2 Division of liquid sample in micro wells

Liquid sample division experiments with food dye are performed on customized pressure jig. 15㎕ of green food dye is injected into micro fluidic platform through inlet and after injection, pressure force applied on the center of device with transparent square shape suppressor. Approximately, 2㎕ of liquid sample is trapped in micro well array(each micro well have 0.108nl volume) and other part of sample is stay in non-micro well area or came out through outlet of device. In the device, liquid sample is isolated according to magnitude of pressure force into the micro wells gradually. From 0N of pressure force to 5N, degree of division is increase and maximum force applied, liquid sample is fully isolated between each other wells around (Figure 2.2). In condition of thin wall structure than 25㎛ thickness, PDMS wall is deformed after full

division causing leakage of liquid sample to perimetric wells.

With structure of micro well array and wide micro fluidic channel, the deformation of each channel wall part is attended and from this deformation, full isolation of liquid sample is not occurred in side area of micro well array. Nevertheless, well structure is fabricated in both side area to facilitate uniform liquid patterning and prevention of air bubble outbreak caused by different surface character between liquid samples to plane surface and well structure surface.

With this characteristic of platform, isothermal digital RPA is performed without interaction between each other micro wells (Figure 2.3).

2.3 Isothermal DNA amplification in platform

Isothermal DNA amplification in the platform is performed with RPA exo kit (TwistDx) and extracted generic DNA from human breast cancer (MDA-MB231) and human colon cancer (SW620). Mutated TP53 DNA (Tumor suppressor DNA) is employed for target DNA and the primer and probe for real time detection are designed according to the mutation of DNA sequence (The adenine mutated from 839th guanine of MDA-MB231 derived TP53 and the

thymine mutated from 925th cytosine of SW620 derived TP53). FAM probe is designed for detection of TP53 extracted from MDA-MB231 and Cal Red Flour 610 probe is designed for detection of TP53 extracted from SW620. Sequence for primers, FAM probes, Cal Red Flour 610 probes and template can be found in table S1. The sequence of primers and probes are designed according to the protocol of the RPA exo kit and the RPA publication [30] and ordered through Macrogen and Biosearch Technologies. And all the images of fluorescence signal are taken with fluorescence micro scope (Nikon Tie, 10x).

For the detection of mutated TP53 in the micro fluidic platform, RPA mix (2x reaction buffer 25㎕, 10mM dNTPs 1.7㎕, DNA free water 5.7㎕, 10x probe E-mix 5㎕, 10㎛M primer mix 4.2㎕, 10㎛M probe mix 1.2㎕, 20x core reaction mix 2.5㎕, 50x exo 1㎕, 280mM MgOAc 2.5㎕) and templates(5.0㎕ of TP53 extracted from MDA-MB231 and SW620 with 108 templates/㎕ concentration, respectively). DNA amplification process is performed in incubator with 39℃ for 40min after injection of mixture and application of pressure force on the device.

For the reaction time experiments, same condition of liquid samples are used to obtain amplifying fluorescence image as amplification time increase. The images are taken in 39℃

incubator with 15 min intervals during 30min. Analysis of fluorescence signal is performed with Image J functions to obtain numerical datum for transformation of light signal to graphical figures.

2.4 Quantification of ctDNA

In the initial template variation experiments, different composition of RPA mix is employed for the reason of high concentration of initial template concentration and extended amplification time. With exo RPA mix (two RPA exo enzyme pellets, 10㎛M primer mixture 4.2㎕, 10㎛M probe mixture 1.5㎕, DNA free water 12.84㎕, rehydration buffer 29.5㎕, MgOAc 5.0㎕) and templates(various volume of TP53 extracted from MDA-MB231 and SW620 with 1010 templates/㎕ concentration, respectively), amplification experiments are performed in 39℃ during 40 min. For each template concentration, control(0 templates/㎕), 100, 101, 102,104, 106 templates/㎕, is performed in the platform and captured with 10x array image. 2000(10% of whole micro well array) micro wells are taken with fluorescence micro scope in the end-point of amplification process. Also, for quantification of fluorescing wells, same condition of micro scope

is employed to count the number of wells.

The criteria about positive or negative of fluorescing well is determined with the numerical value of fluorescence intensity. After comparison of fluorescence intensity with initial state of micro wells and the fluorescing wells after amplification process, positive wells (count as fluorescing well after amplification) appear with more than doubled intensity signals. After positive well count, the numbers of fluorescing wells are arranged according to the initial template concentration conditions.

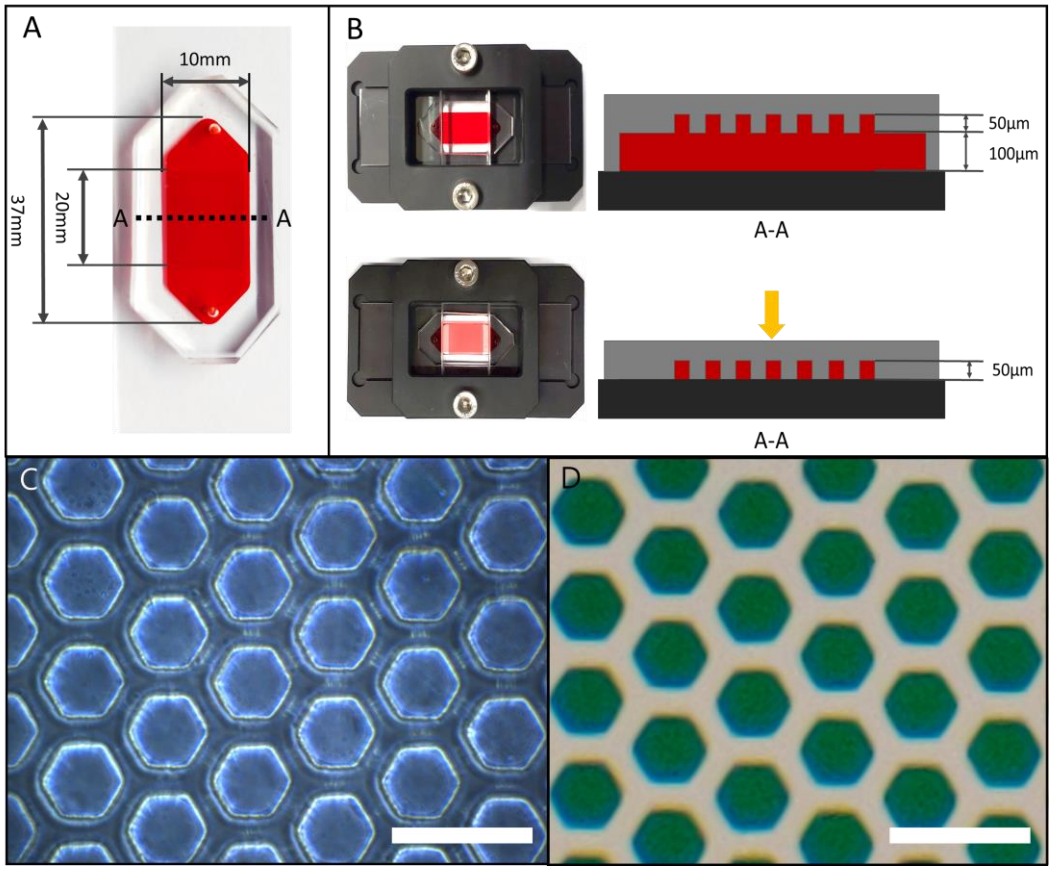

Figure 2.1 Schematic of micro fluidic device and honey comb structure in micro well array. (A) Top view of the photograph of PDMS device. In the center of micro fluidic platform, 20,000 micro wells are located in 20mm length and 10mm width square area. (B) Figure of device before pressing and after pressing. By applying pressing force, PDMS device attached on glass coverslip and the liquid sample is isolated in micro wells. Rest sample came out through inlet and outlet or stay in plane channel area. (C)

Photograph of micro-well structure with optical microscopy. The diameter of inscribed circle of hexagonal well is 50 μm. (D) Isolated dye in micro well array. Scale bar = 100 μm.

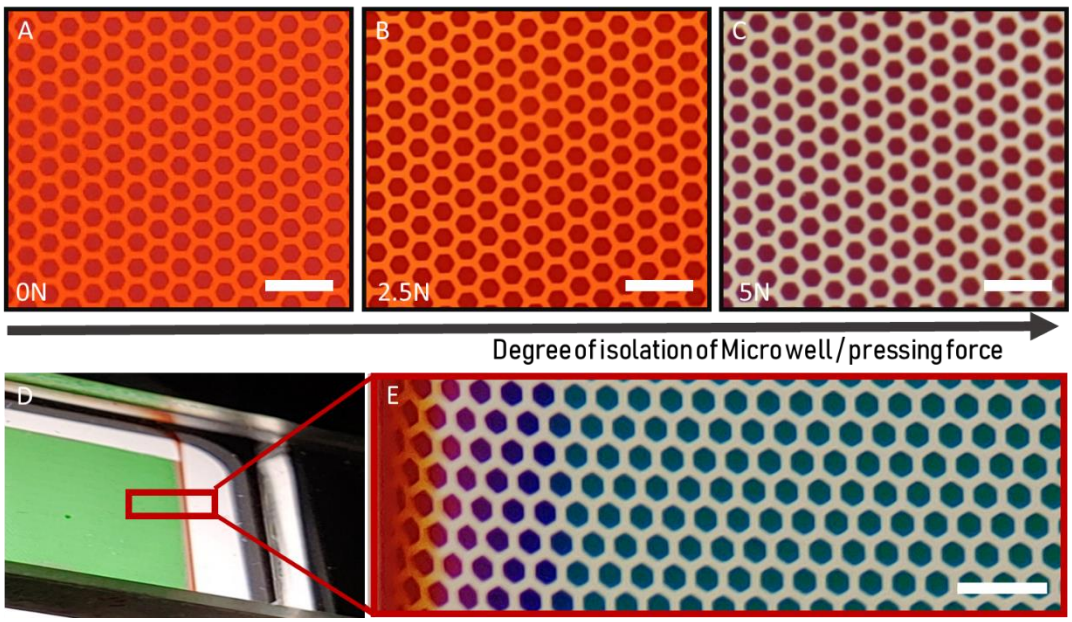

Figure 2.2 Feature of isolation process and in the edge of micro well array. (A) Photograph of micro well array without pressure. (B) Photograph of micro well array after pressed with 2.5N pressing force. (Half-isolated state) (C) Photograph of micro well array after fully pressed with 5N force. (Full-isolated state) (D) Injection of different dye after full pressure to analyze the degree of isolation of micro well array. (E) Photograph of the edge section of micro well structure. Micro wells are fully isolated locate on 400 μm from the side wall of micro fluidic channel. 92% of whole micro

wells are fully isolated under full pressing force. Scale bar = 200 μm.

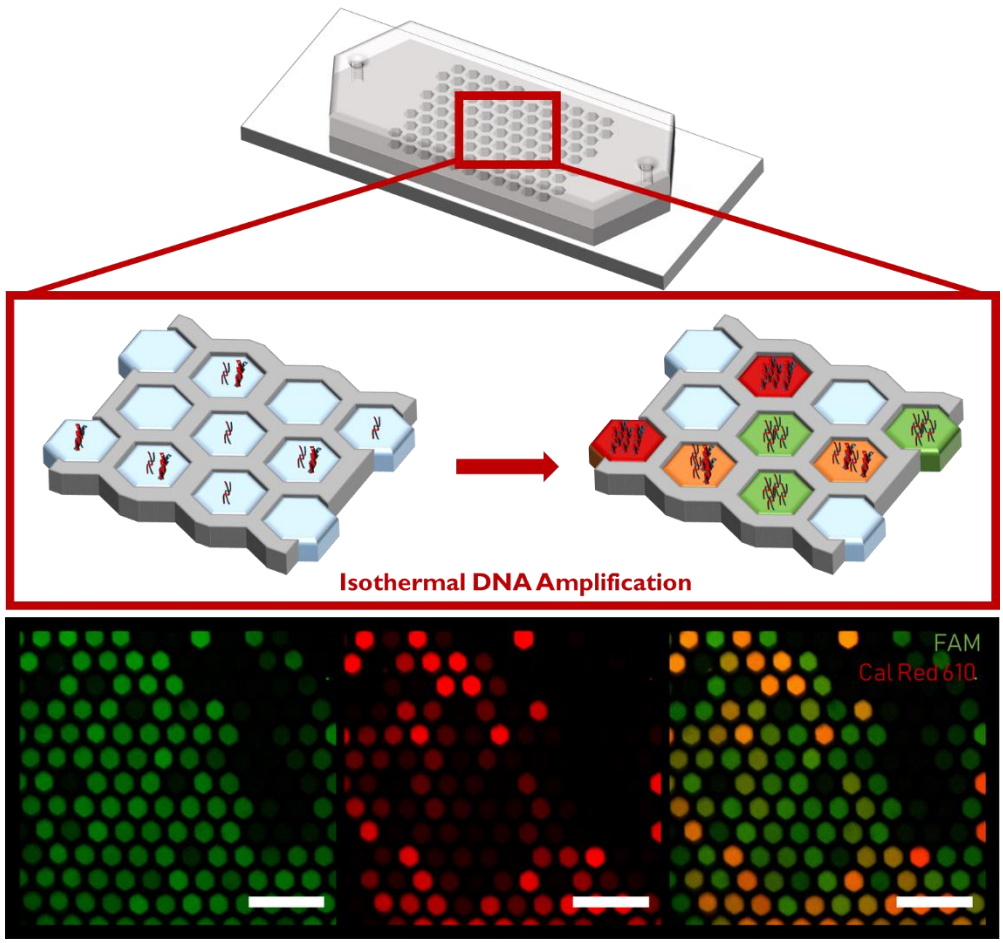

Figure 2.3 Schematic of isothermal digital PCR process in micro fluidic platform. In each 20,000 micro wells, reagents and generic DNA extracted from tumor cell perform isothermal DNA amplification process. End-point image after DNA amplification

process appear as the below figure. From the left to middle, each color of fluorescence represent different DNA amplifications and from the right image, amplification processes in this platform progressed independently in fully isolated micro wells without the interaction of surrounding wells.

Chapter 3. Result

3.1 Detection of tumor DNA through digital RPA

The device filled with mixed RPA exo kit and pressure force is applied on the center of the device. Customized pressure jig is employed to apply pressure force on micro well array area, and the live cell imaging system is employed to make proper temperature conditions and imaging conditions. Amplification is performed for 40mins in 39℃ after injection of reagent mixture and force application. After amplification processes, fluorescence signals are detected in each sealed micro wells. Isolated DNA sample in each micro well contain templates and amplicons respectively, and both two fluorescence signal is detected in some of the micro well according to the condition. From the initial state of amplification process, micro wells are sealed perfectly until the final state of DNA amplification and with this condition, amplicons and probes are fully isolated in each micro wells without leakage.

TP53 extracted from breast cancer (MD-AMB231) signified with FAM probe (green) and other TP53 extracted from colon cancer

(SW620) signified with Cal Red Flour 610 (red) (Figure 3.1 B, C). Each DNAs are amplified independently. In case of the wells which have both fluorescence signals in same well, both TP53s are captured in micro well and the amplification process is applied to both templates.

With merged image of fluorescing wells with bright field image, isolation of micro wells are detected obviously (Figure 3.1 E). Fluorescing liquid samples are trapped in sealed micro wells only and non-fluorescing wells are remain with just bright field image.

3.2 Real time detection of fluorescence signal

Amplification study to measure fluorescence intensity is required for quantification of ctDNA. By employing high initial template concentration condition (10^8 templates/㎕), the real-time measurement of fluorescence intensity is performed. During 30min amplification time, the platform is placed under 39℃ condition with real time imaging system. From the initial state to final state, fluorescence intensity increased as time variable increased. At first state, fluorescing wells are not detectable until the sufficient amplification. In second state (after 15min), fluorescence signal is

detected in several micro wells with more than doubled numerical value of intensity and with the visual value, also. At the final step (after 30min), intensities are increased more than triple or quadruple numerical value comparing with initial state. Also, fluorescence signal values are doubled from second state. Relative numerical values are shown in the graphs with time variations and according to the distance from the standard point of measurement (Figure 3.2).

3.3 Quantification with fluorescing micro wells

Quantification of DNA sample with various initial template concentration conditions are performed with the purpose to confirm about validity and effectiveness in actual clinical conditions. Previous researches refer that ctDNA concentration in the blood sample of cancer patients is extremely low concentration in specific cancers such as colorectal cancer and breast cancer [31, 32]. Based on the datum of previous researches, initial template concentration condition is confirmed from 0 copies/㎕ to 106 copies/㎕ and experiments are performed from highest concentration condition to lowest concentration condition. The lowest template concentration reflect the actual concentration of

ctDNA in blood sample of breast cancer patient.

With these conditions, each experiments are performed with same temperature condition and amplification time. All the reagents are amplified for 40min in 39℃ after injected into device and all the images are taken with Nikon Tie with multi array imaging function. Each image contain 2,000 wells approximately and this wells are correspond to 10% of whole wells in micro well area.

After amplification, fluorescing wells are detected according to the template concentration conditions and in the lowest concentration condition, maximum 20 fluorescing wells are detected. All the image of experiments show the number of fluorescing wells are increase or decrease according to the conditions and datum from quantification of DNA samples are arranged with graph (Figure 3.3).

Based on the result of the experiments, the lowest template concentration which can detect with this platform is 1 copies/㎕. With this result and the actual data of clinical research, the possibility of application of this platform into clinical situation is proved [9].

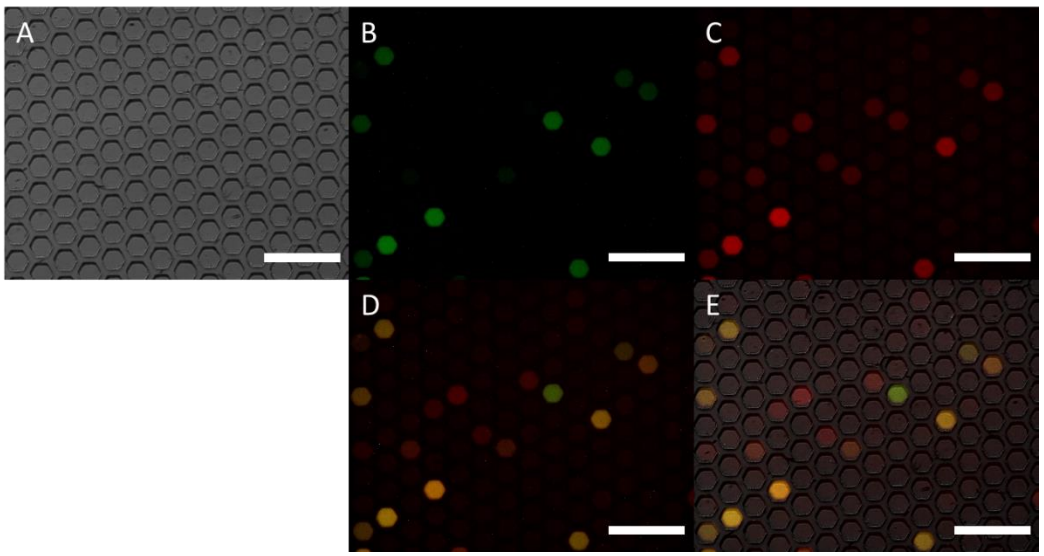

Figure 3.1 End-point fluorescence image of target DNA after isothermal DNA amplification (TP53 extracted from SW620 and MD-AMB231). (A) Bright field image of micro well area. (B) FAM fluorescence image of TP53 extracted from MDA-MB231 with initial template concentration = 10^8 copies/μl. (C) Cal Red Fluor 610 fluorescence image of TP53 extracted from SW620 with initial template concentration = 10^8 copies/μl. (D) Merged image of both fluorescence images. (E) Merged image with gray scale image. Amplification of DNA is occurred in fully isolated micro wells without interaction with other micro wells surround. Scale bar = 200 μm.

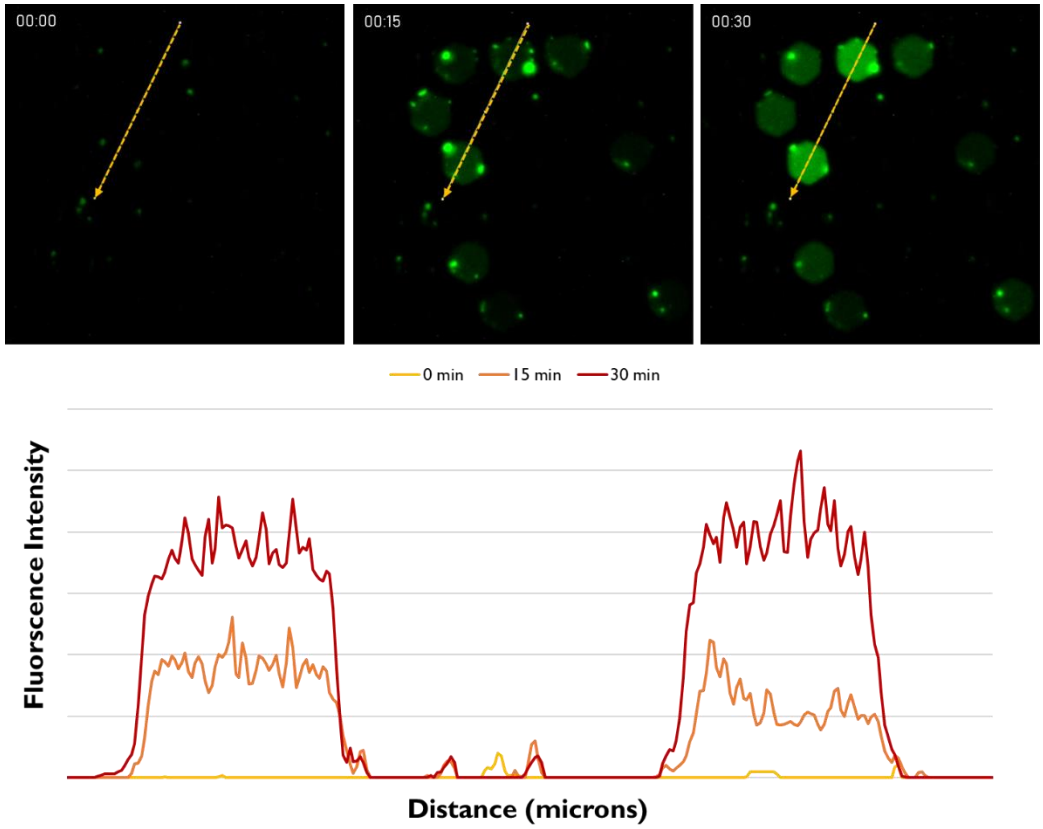

Figure 3.2 Real time fluorescence signal image and graphical figure of fluorescing micro wells on the process of DNA amplification. Each image show fluorescence signal increasing during amplification process. From beginning of amplification to 30 min after, fluorescence signal numerical value increase dramatically. With the standard of fluorescence signal value, wells with more than doubled fluorescence count as fluorescing wells after 30min.

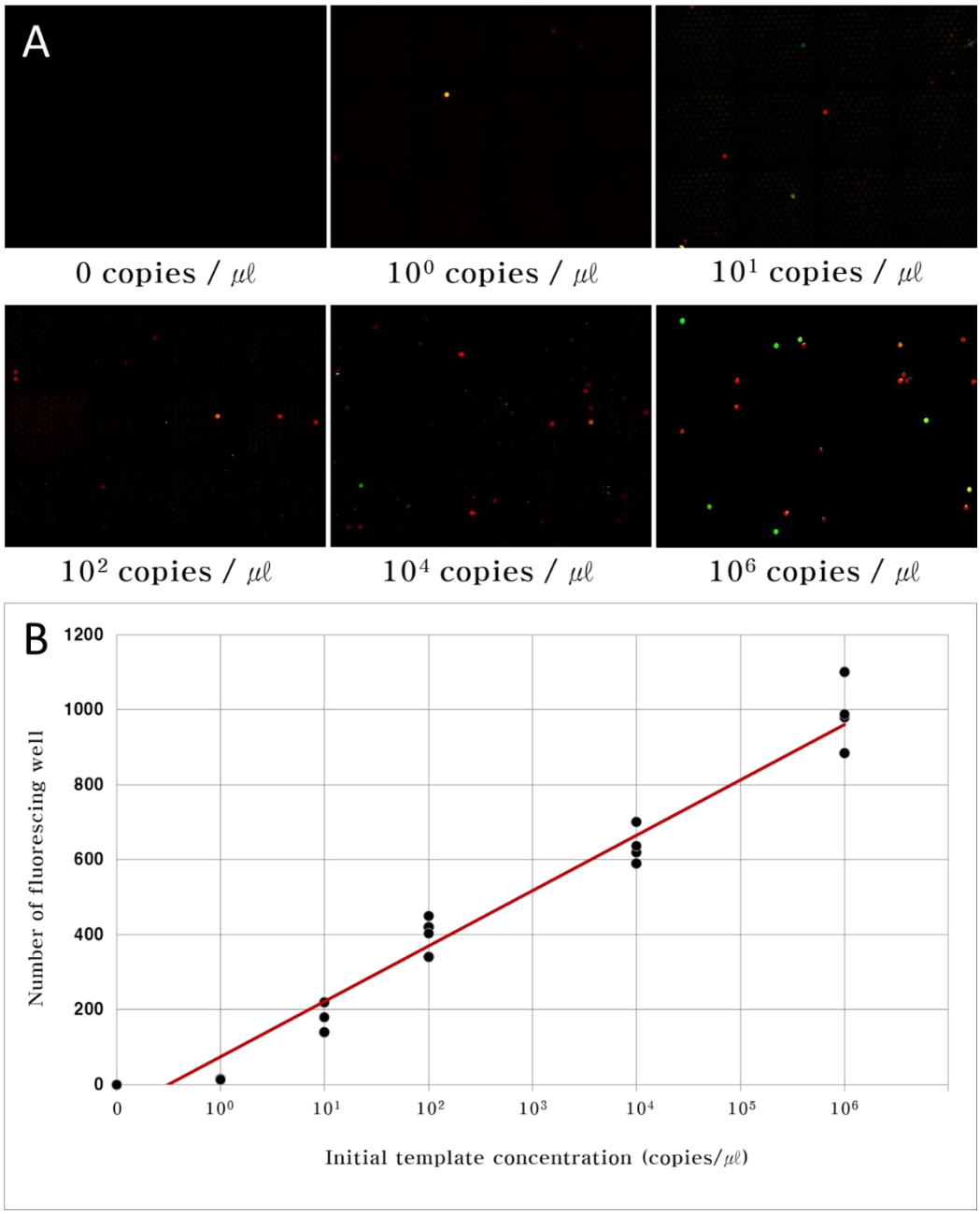

Figure 3.3 End-point images of fluorescence detection in microfluidic platform after digital RPA process with diverse initial

template concentration conditions and quantification of digital RPA with graphical figure. (A)Each image represent the result of different initial template concentration conditions after digital RPA process in microfluidic platforms. According to the increase of initial concentration of templates, the fluorescing wells in end-point increase. All the images contain 2,000 wells (10% of whole micro wells) and the wells which have mutated DNA in initial state appear with high intensity of fluorescence in the final state. During 40min amplification at 39℃, each DNAs isolated in the micro wells amplified with the increase of fluorescence intensity without interaction between surrounding wells. (B)According to initial template concentrations, the number of fluorescing wells increased. The sections from the control (0 copies/㎕) to 103 copies/㎕ condition stand for extremely low concentration of ctDNA in actual cancer patient blood sample and robust detection in this sections signify the possibility of usage on the site.

Chapter 4. Discussion

This study present isothermal digital PCR platform based on microfluidic system with simple and robust sample isolation and colony formation. To quantify mutant gene samples, DNA amplification technique is required with high throughput systems arise from extremely low concentration of the mutated DNA [33-36]. Robust and sensitive detection and quantify assay required for the cell free or circulating tumor DNA. And conventional platforms such as droplet PCR, digital PCR on microfluidic devices reflect these characteristics sufficiently [20, 28].

But although this advantages of conventional platforms, conventional researches require high cost equipment, well-trained technicians and time consuming caused by complex process and property of PCR technics. Even with microfluidic systems, complex structures are required to form thousands of, ten thousands of droplet or isolated samples and these structures make distance between microfluidic digital PCR platforms from POC platforms.

Unlike conventional digital PCR platforms, this study present platform facilitate easy-to-use, simple, fast sample isolation and

colony formation and with RPA technique, fast isothermal DNA amplification process is performed in low temperature. Also with 20,000 micro wells in the middle of microfluidic channel, high throughput screening is possible with small area. Using this platform, next research will be more fine size micro structure for higher throughput and exquisite isolation of single particle. Based on the previous research, the isolation degree of particle is decided according to the volume of micro wells and with smaller well will contain smaller number of particle [29]. Also, with sufficient quantify assay methods or system, this platform have lot of possibility to employed as one of the point-of-care (POC) platforms.

Chapter 5. Conclusion

By apply pressure force with simple motion, liquid sample is isolated in 20,000 micro well array and isothermal digital DNA amplification process is performed in this platform. With this high throughput screening platform, detection of mutant DNA extracted from colon and breast cancer cell is performed with the mimicked environment from the actual condition of circulating tumor DNA in

cancer patient blood. Through this study, this platform demonstrate the potential of microfluidic systems use for digital PCR process without degree of complexity and time consuming and the possibility of usage in clinical situation or as a platform for point-of-care system.

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초 록

혈액 내 순환 종양 유전자의 정량 검출은 기존의 항체 기반 바이오마커 검사나 조직 검사에 비하여 암 검출을 침습성 검사 혹은 개개인의 환자에 따라 변수가 결정되는 일 없이 가능하다. 이러한 유전자 기반 바이오마커의 검출을 위해서 많은 유전자 관련 기술들이 중합효소연쇄반응(PCR) 기술을 기반으로 활발하게 개발되었다. 특히, 디지털 중합효소연쇄반응은 정량 분석에 특화 되어있는 기술로, 전체 표본에서 농도가 매우 낮은 유전자 표본을 검출할 때 일반적으로 사용된다. 하지만 기존의 디지털 중합효소연쇄반응 과정은 일반적으로 복잡한 과정으로 인하여 높은 가격의 장비, 숙련된 기술자 그리고 많은 시간 소모를 동반한다. 이로 인해, 기존의 디지털 중합효소연쇄반응을 미세 유체 시스템을 통하여 대체하려는 연구가 현대사회에서 진행되고 있다. 이 연구에서는 미세 유체 시스템과 재조합 효소 중합효소 증폭(RPA)라고 불리는 등온 유전자 증폭 기술을 통하여 혈액 내 순환 종양 유전자의 정량 검출을 고가의 장비와 많은 시간 소모 없이 가능한 미세 유체 플랫폼을 연구하였다. 등온 유전자 증폭 기술을 통하여 고온에서 진행되는 기존의 중합효소연쇄반응의 한계점을 해결하였다. 또한 설계된 플랫폼을 통하여 디지털 중합효소연쇄반응에서 중요한 표본의 콜로니 구성을 정확하고, 빠르며, 간단하게 진행할

수 있다. 이 플랫폼에서는 간단한 압력을 가해 유체 표본을 20,000개의 미세 웰 구조에 분리해낼 수 있으며, 또한 암세포에서 추출한 유전자를 통하여 실제 환자에서의 혈액 내 순환 종양 유전자를 모사하고, 혈액 표본에서 매우 낮은 농도를 가지는 순환 종양 유전자의 환경을 유사하게 구현하였다. 추출된 변형 유전자는 설계된 프라이머, 프로브를 이용하여 미세 유체 플랫폼에서 성공적으로 검출이 가능하였다. 이 연구에서는 이를 통하여 혈액 내 순환 종양 유전자의 정량 검출이 확실하며 사용이 용이한 플랫폼을 현장현시검사(POC) 플랫폼으로 제안한다.

주요어: 미세 유체 소자, 디지털 중합효소연쇄반응, 혈액 내 순환 종양 유전자, 현장현시검사, 진단학
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