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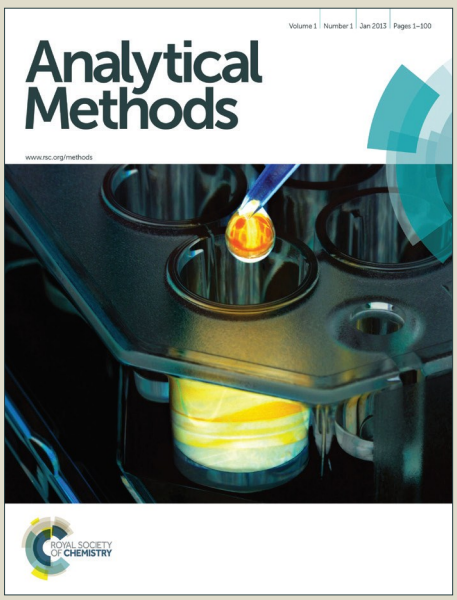

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ARTICLE TYPE

A localized temporary negative pressure assisted microfluidic device for detecting keratin 19 in A549 lung carcinoma cells with digital PCR

Qingchang Tian [a,b], Qi Song[a], Yanan Xu[a], Qiangyuan Zhu[a], Bingwen Yu[a], Wei Jin[a], Qinhan Jin[a] and Ying Mu*[a]

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[a]*Research Center for Analytical Instrumentation, Institute of CyberSystems and Control, State Key Laboratory of Industrial Control Technology, Zhejiang University, Hangzhou 310058, Zhejiang, P. R. China.*

[b]*College of Life Sciences, Zhejiang University, Hangzhou 310058, Zhejiang, P. R. China.*

**Author to whom correspondence should be addressed. Electronic mail: muying@zju.edu.cn; Fax.: +86 571-88208382; Tel.: +86 571-88208383*

Digital polymerase chain reaction (dPCR) has been play a major role in biological research, especially been an expert in counting of single nucleic acid molecule. Here we present syringe filter-like microfluidic device to realize sample loading, encapsulation, moisturizing and running dPCR. The gas-permeability of polydimethylsiloxane (PDMS) is utilized for sample loading under negative pressure. The air in chambers is evacuated to the negative pressure side, and brings the sample solution into chambers. We also add a vaporproof-layer (VPL) in the chip to moisturize or restrain evaporation caused by the gas-permeability of PDMS under thermalcycling. Digital PCR is applied to test keratin 19 on this microdevice with 650 chambers ,each having a volume of 6.28 nL, using the cDNA from A549 cell line. The results show a linear regression under five dilution concentrations, and demonstrated the robustness of the dPCR chip. This device is easy to be fabricated without multiple overlay exposure or high alignment precision, and should be a tool for biology research.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, which is a heterogeneous disease with a variety of histopathological types and subtypes. Keratin 19 (KR 19) is applied to detect tumour cells in lung cancer patients with PCR[1], and seems to be the most sensitive and reliable tumour marker in cancer patients for predicting disease[2, 3].

Digital polymerase chain reaction (dPCR) has been playing a major role in biological research[4]. With highly sensitive nucleic acid quantification, dPCR methods have been applied to assess the allelic imbalance [5-8], measure the gene copy number variation [9, 10], test the fetal nucleic acids in maternal plasma for non-invasive prenatal diagnosis [8, 11] and quantify the amount of the transgenic event in genetically modified organisms [12, 13].

For achieving to count the number of 'positive' reactions versus 'negative' reactions in dPCR, the sample is diluted and partitioned into hundreds or even millions of separate reaction chambers so that each contains one or no copies of the templates. Since Vogelstein and Kinzler [4] accomplished dPCR with a 96-well plate for a long time, it was troublesome to implement dPCR in hundreds or even millions separate chambers using 96 or 384-well plate. Advances in microfluidic have assisted to miniaturize the dPCR reactive volume to nanoliter or even picoliter scale, and made dPCR easier to perform.

There are several microfluidic formats with subtle and complex operating system to enforce the sample solution into separate small-scale reactor and hold the PCR solution calm under thermal cycle. Slipchip[14, 15] relies on two close contacted plates shaping interlinked channel for sample injection and 'slipping' to compartmentalize the nucleic acids. Openarray[16] is fabricated with 3072-wells of hydrophilic interior and hydrophobic exterior to prevent cross-contamination and evaporative loss during temperature cycling. Stephen Quake's group[17-19] developed complex microfluidic large scale integration chips with thousands of microvalves and control components. The integrated fluidic circuit (IFC) chip has supported the implementing of dPCR in many areas of biology [12, 20-22]. The other laboratories have developed kinds of microfluidic formats for dPCR, such as, a spinning disk platform[23], megapixel digital PCR[24], femtoliter array[25], and emulsion PCR or droplets[26, 27]. Microfluidic devices for dPCR undergo troublesome sample loading and compartmentalization, also avoid cross contamination and evaporative loss during temperature cycling. Usually the sample loading has been attained by positive pressure

with pumps and microvalves [24, 25] or by hydrophilic interior [16].

High gas permeability is one of properties of PDMS[28], which make it easy to remove air out of PDMS microfluidic device. Utilizing the high gas permeability of PDMS to provide sample loading power, some works reported negative pressure or vacuum-assisted sample loading for facilitating the chip fabrication and operation processes. Zhu[29] reported a Self-priming compartmentalization chip in which the energy for the pumping was pre-stored in the degassed bulk PDMS. The chip need to be placed in a vacuum to be pre-vacuumed. When the chip is brought back to the atmosphere, the re-dissolution of air through the microchannels walls provides a kinetic energy for the solution to move into the channel.

Although the vacuum-assisted sample loading is a convenient way, there are still practical limitations[30]. The device needs to be stored in a vacuum device for more than 30 min to evacuate the air in PDMS or sealed shrink-wrap vacuum packaging. When the device is exposed in the atmosphere, it should be used immediately in case its pumping ability decays immediately with time. Martin Kolnik[31] developed a novel microfluidic cell culture device which cell loading was achieved by generating a localized temporary on-chip vacuum in channels directly adjacent to the cell culture chambers and the air in chambers was evacuated into channels. When the air in chambers passed through the PDMS into channels, the cells were brought into the chambers. It is a controllable and handy way for sample loading in PDMS. In this paper, we adopted this localized temporary on-chip negative pressure for dPCR reagent loading.

The permeability of PDMS is a double-edged sword, facilitating sample loading while bringing evaporation. Water in reaction system under 95 ℃ in dPCR progress become water vapor and water vapor escape the PDMS to bring evaporation loss. The evaporation in chip not only brings about dryness in some chambers but also reduces the efficiency of PCR, and obtains an unfaithful dPCR result finally. Under nanoliter or picoliter scale, evaporative loss of water in bio-reaction is fatal. It is restrained by surface treatment to eradicate the gas-permeability of PDMS[24], or microvalves and heavy-press to avoid evaporative loss during temperature cycling in chip[25]. The self-priming compartmentalization chip (SPC chip)[32] is treated with a vapor of timethylchlorosilane to form a nano-waterproof layer.

In this paper, we developed a novel microfluidic device to avoid evaporation loss and perform dPCR. This device adopted the gas-permeability of PDMS for sample loading and hydrophobicity of oil for compartmentalizing to prevent cross-contamination. And it was added a protected layer VPL to moisturize and prevent evaporative loss caused by the gas-permeability of PDMS under thermalcycling. In this way, not only sample loading is facile without microvalves or heavy equipment, but also evaporative loss caused by the breathability of PDMS is reversed by moisturizing.

Experimental

1. Design and fabrication

The lamina-chip layer (LC) and VPL are loaded sample liquid by a syringe filter-like microfluidic device (μfilter) with helical channel to provide localized temporary on-chip negative pressure. The LC and VPL are thin layers with chambers.

The microfluidic device patterns were designed via Corel DRAW X4, printed on transparency films, and exposed onto photoresist (SU-8, MicroChem) to form the mold on silicon wafers. The μfilter mold was prepared by spin-coating photoresist (SU8-3025) onto 4-inch silicon wafers (1000 rpm for 30 s) to create an 70 μm high helical channel. After the wafers with photoresist were soft baked at 95 ℃ for 30 min, the patterns of μfilter were exposed onto them by ultraviolet light. Then the exposed wafers were placed on a 95 ℃ hot plate for 30min to take post exposure bake. And then the mlods were developed and hard baked at 200 ℃ for 60 min. The LC and VPL molds were prepared by spin-coating photoresist (SU8-3050) onto silicon wafers (1000 rpm for 30 s) to create an 115 μm thick layer. After softer baking, a second layer of SU-3050 was overlapped on the first layer. After exposure and development of the two SU8-3050 layers, 230 μm high molds were built on silicon wafers.

The microfluidic devices were made from PDMS (GE RTV 615) that is a two component PDMS elastomer. Polydimethylsiloxane (PDMS) was obtained from Dow Corning (Midland, MI, USA). The molds were treated with a vapore of trimethylchlorosilane (Aladin) before use to prevent adhesion of PDMS. PDMS mixture (10 A: 1 B) was poured on the μfilter mold to form a 5mm thick for mechanical stability. And a 500 μm-thick layers were created by spincoating PDMS mixture (10A: 1B) on the LC and VPL molds. Then the molds were baked on a hotplate at 80 ℃ for 45 min, the PDMS block on the μfilter mold was peeled off and punched holes. Two μfilter PDMS blocks constructed a empty μfilter. The thin LC or VPL layer were peeled off and clamped between μfilter like a filter membrane.

2. Microchip operation

Silicone oil (50 cst) was used for blocking and compartmentalizing sample solution. Four gram of silicone oil was mixed with 1.1 g of uncured PDMS(10 A : 1 B), and then the mixture was vortexes and degassed in a vacuum device.

One of the two μfilter PDMS blocks connected with a syringe, and clamped the LC or VPL layer with the other μfilter PDMS block, ensured that the chambers in the LC and VPL were back to the μfilter PDMS block with syringe. When the syringe was pulled, air in the chambers was permeated the PDMS into syringe with the two air pressure differences, and brought sample solution into the chambers. Then excess solution was taken off from LC and blocked by silicone oil.

3. Digital PCR

The human A549 lung carcinoma cells were collected to extract total RNA using AxyPrep Multisource Total RNA Miniprep Kit (Axygen Biosciences). Then the total RNA was reverse transcribed into cDNA. The template cDNA from A549 cancer cell line was diluted at 5 orders. The reaction mix (20 μL) was prepared in a 0.2 mL tube which was comprised with Taqman Gene Expression Master Mix 10 μL, Taqman Gene Expression Assay 1 μL, cDNA solution 6 μL and RNase-free water 3 μL, and loaded into chip as mentioned above. The Taqman Gene Expression Master Mix (4369016) and Taqman Gene Expression Assay (ID HS00761767_s1, KR19) were obtained from Life Technologies (Applied Biosystems) (Fostor city, CA). The

primers and probes in the Taqman Gene Expression Assay was used to detected single exon of human gene Keratin 19, and the amplicon length is 116 bp. The experiments were repeated three times to ensure the robustness and repeatability of our platform. The chip was placed on PCR device (MGL96G, Long Gene) and pressed tightly by the hotlid to run thermalcycling program: 40 two-step cycles (20 s at 95 ℃ and 60 s at 60 ℃) with an initial denaturing step (5 min at 95 ℃).

4. Data acquisition and analysis

After run thermalcycling program, the chip was scanned by the Maestro EX IN-VIVO Image System (CRI Maestro). Degraded Taqman probes in positive chambers were excited at 455 nm and the emitted 518 nm light was detected by CCD through a 495nm long filter. The images were analyzed by the Image-Pro Plus V 6.0 software to count the number of positive chambers.

Result and discussion

1. Principle of the sample loading and fabrication of μfilter microdevice

The microfluidic chips are fabricated by soft lithography techniques. The μfilter microdevice was designed to achieve negative pressure sample loading. Because of the gas-permeability of PDMS, air can permeate into the PDMS from higher pressure side, and also be forced out PDMS into lower pressure side. Under a continuous negative pressure, all air in higher pressure can go through the PDMS to the other side. Based on the above principle, we designed the μfilter microdevice to provide localized temporary on-chip negative pressure for the sample loading. As fig. 1A shown, the annular duct (200 μm in width, 40 μm in depth) in the μfilter connected to the ports (0.5 mm) punched in the PDMS layer. In one part of the μfilter, a syringe was connected to the port, and in the other part of the μfilter, there were a sample port for loading sample and a oil port for sealing sample. The annular duct in a 20 mm diameter area covered the all chambers in the LC. The reaction chambers in the LC were cylindrical with 200 μm diameter and 200 μm depth, and the distance between two chambers was 200 μm too. There were 650 reaction chambers in the LC in a square area 15.0 mm×15.0 mm. and the diameter of chambers was 100 μm in the VPL with a 17.0 mm diameter circular area. The LC can be covered entirely by the larger VPL (Fig. 1B).

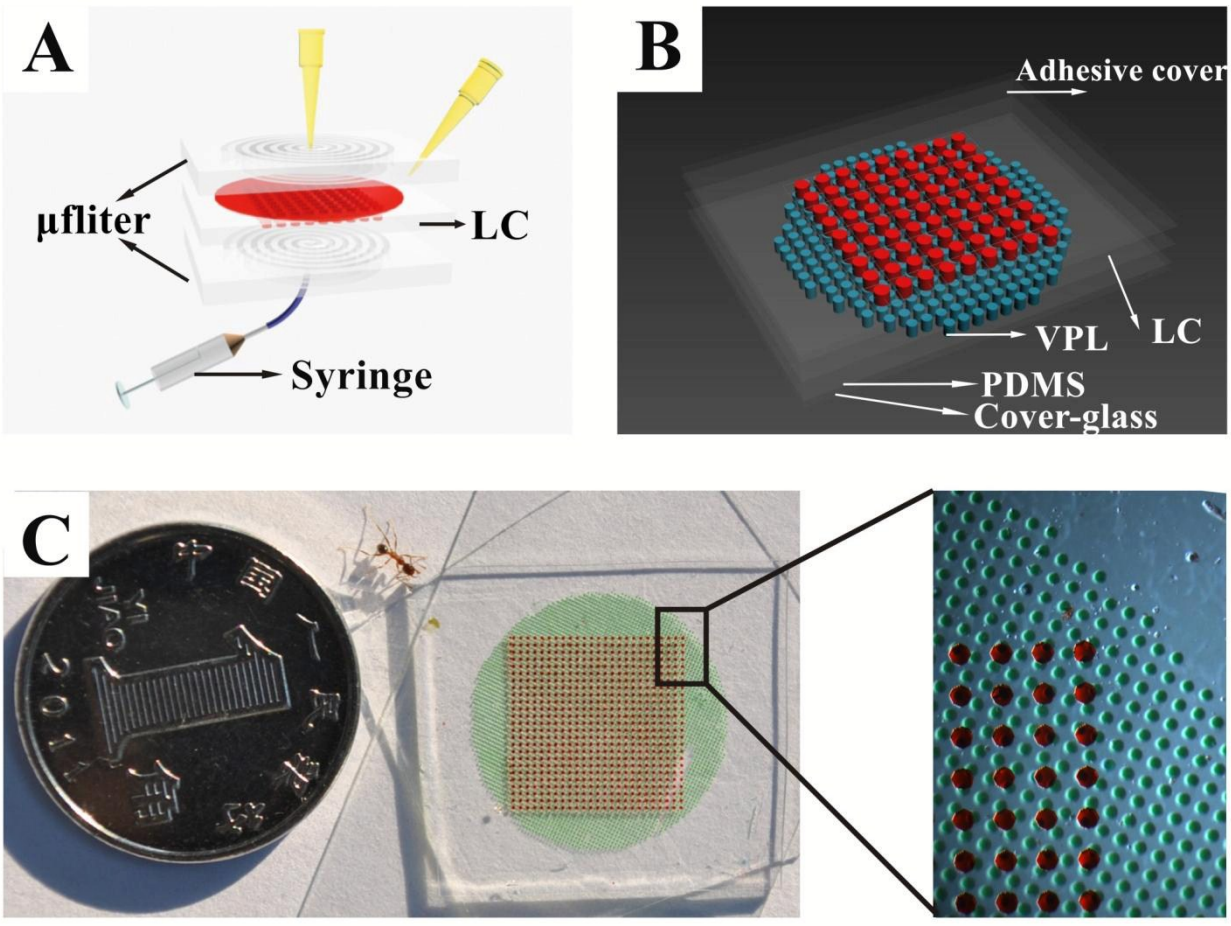

Fig. 1. Schematic drawing shows the design and mechanism of the μfilter microdevice. (A): Schematic diagram of the microfluidic chip, showing the process of reagent loading into LC via μfilter microdevice. (B): Schematic of the reagent loaded chip. It contains reagent loaded LC (red), water loaded VPL (blue), PDMS on cover-glass and optical adhesive cover. (C): Photograph of the prototype device.

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When the LC was pasted on the μfilter, the annular duct in the μfilter was sealed up. Once drawing out the syringe, air pressure in the sealed annular duct was lower than atmosphere pressure in chambers for sample loading in the LC. Under the localized temporary negative pressure, air in the chambers was permeated into μfilter and brought the sample solution into each chamber.

The sample solution was discretized by the negative pressure from syringe. As showing in Fig. 2A, two μfilter PDMS blocks clamped LC to constructed an empty μfilter for sample loading. The reagent solution was dispensed on the injection port, then drawing out the syringe, air in the chambers was evacuated into syringe through the PDMS and brought sample solution into the μfilter (Fig. 2B). Then sample solution was brought into the chambers under the sustained negative pressure. Once the sample was completely primed into the chambers, the silicone oil was dispensed into the oil port (Fig. 2C). Silicone oil has affinity with PDMS and repellency with water. Once the oil enter the port, it could invade into the gap of two layers of PDMS (Fig. 2D), propel excess solution aside, and isolated sample solution from the environment (Fig. 2E). Next a sizeable Optical Adhesive Cover (ABI, 4312063) cover the area, and the thin chip was peeled off. The VPL was loaded deionized water as mentioned above. In the end, the LC was taken down from the μfilter and pasted on VPL to form the LC-VPL (Fig. 2F). The LC-VPL was placed on a cover-glass for stabilizing and avoiding evaporation and assembled to a lamina-chip.

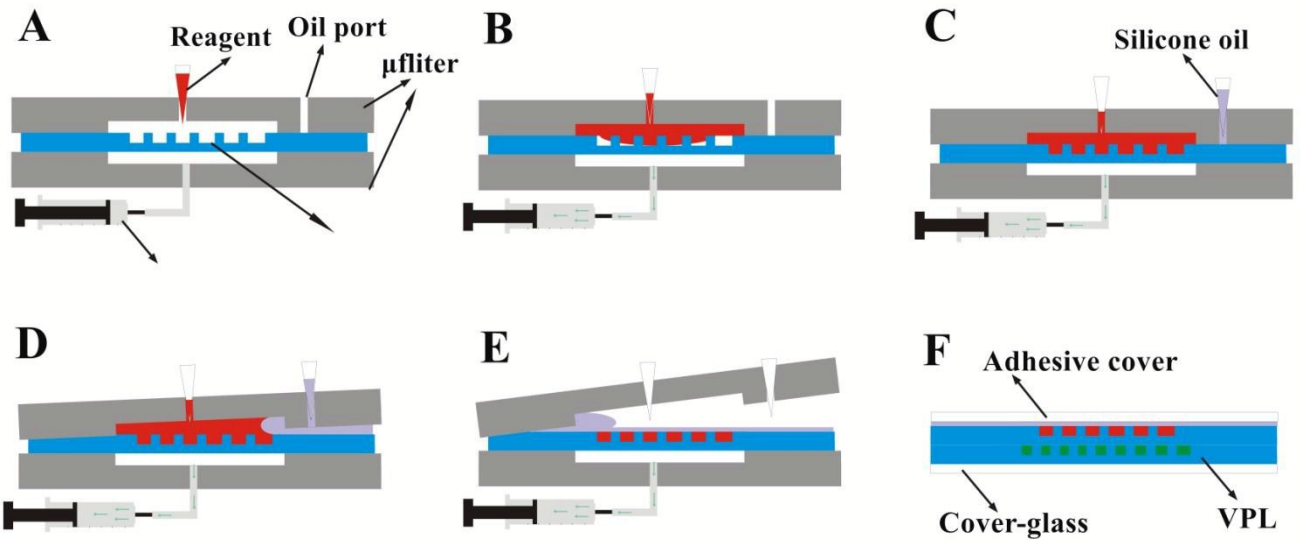


Fig. 2. The principle and operation procedure of the negative pressure assisted lamina-chip. (A): The LC was clamped by the μfilter for reagent loading. (B): Drawing out the syringe, air in the chambers was evacuated into syringe through the PDMS and brought reagent solution or water into the chambers. (C): Chambers in LC were full of reagent solution and then the silicone oil was dispensed into the oil port. (D): Excess reagent solution was carried off from LC by the silicone oil. (E): LC was take off from μfilter and pasted on a PDMS-layer. (F): Cross-section of the lamina-chip.

2. Moisturizing

The breathability of PDMS is a double-edged sword, facilitating sample loading while bringing evaporation. The evaporation in chip not only leads to dryness in some chambers but also reduces the efficiency of PCR, and brings an unfaithful dPCR result finally. Compared to the procedure of integration of the parylene C membrane[24] and low-permeability fluorosilane polymer[32], the workflow of moisturizing is simple and economic. The VPL was employed for moisturizing to LCL. As shown in Fig. 3, after thermal cycle of PCR, the marginal chambers in VPL were evaporated to dryness, while it was different near centre. The evaporation in the marginal chambers was much more serious than those in the other chambers. The VPL that was larger than the LCL sacrificed itself and moisturized the all chambers in the LCL to avoid evaporation and ensure the efficiency of PCR in each chamber.

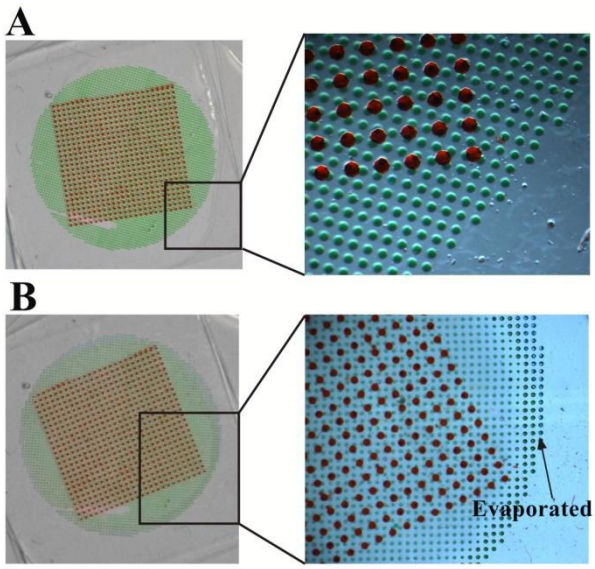
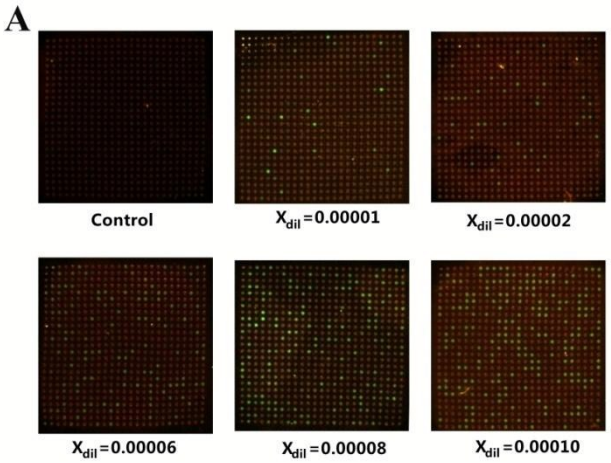


Fig. 3. The evaporation in chip after thermal cycle of PCR. (A): Photograph of the chip before thermal cycle of PCR. (B): Photograph of the chip after thermal cycle of PCR.

3. Quantization of cDNA molecules by dPCR

The LCL-VPL supported by a cover-glass to constitute the lamina-chip for dPCR. The lamina-chip was tested to amplify the oncogene *Keratin 19* in cDNA from A549 cell line by dPCR on a bench-top PCR machine (MGL96G, Long Gene). The cDNA solution was diluted at 5 orders of magnitude from 1×10^{-5} to 1×10^{-4}. The reaction mix (20 μL) was prepared in a 0.2 mL tube which is comprised with the 2× Taqman Gene Expression Master Mix 10 μL, 20× Taqman Gene Expression Assay 1 μL, cDNA solution 6 μL and RNase-free water 3 μL, and loaded into chip as above.

The chip was placed on PCR device and pressed tightly by the hotlid to run thermalcycling program: 40 two-step cycles (20 s at 95℃ and 60 s at 60 ℃) with an initial denaturing step (5 min at 95 ℃). After dPCR thermo cycles, the chip was transferred to the Maestro EX IN-VIVO Image System (CRI Maestro) to take an image of the fluorescence intensity in chambers. After 40 dPCR cycles the fluorescence intensity chamber with zero copies of DNA is significantly weaker than the fluorescence in positive chamber. An image of dPCR on the chip with concentrations of cDNA is shown in Fig. 4(a). As the template concentration increase, the fraction of positive chambers increased. According to the Poission distribution[33] $P(n,\lambda)=(\lambda^n\cdot e^{(-\lambda)})/n!$, the chance of having at least one copy template per chamber is given by $P(n>0,\lambda)=1-P(n=0,\lambda)=1-e^{(-\lambda)}$. Here n is the number of DNA templates per chamber, and λ is the average number of DNA molecules per chamber. So $\lambda=c_0 v\cdot x_{dil}$, and c_0 is the stock concentration of DNA templates, v is the volume of each chamber, and x_{dil} is the dilution factor[29], x_{dil}=1 for the stock solution, x_{dil} =0.5 for a 2-fold dilution of the stock solution, and so forth. While one chamber gives a positive signal, it contains no less than one template. So the observed fraction of positive chambers $f_0=P(n>0,\lambda)=1-e^{(-c_0 v\cdot x_{dil})}$, and $f_0=1-e^{(-c_0 v\cdot x_{dil})}$ can be transformed to $\ln(1-f_0)=-c_0 v\cdot x_{dil}$. The regression curve equation between $\ln(1-f_0)$ and dilution factor x_{dil} shows the a linear variation relationship. Fig. 4B shows a linear regression under four dilution concentrations by plotting $\ln(1-f_0)$ against the dilution factor x_{dil}. The stock concentration of DNA templates was determined from the linear regression, and yielded the stock concentration of DNA templates $c_0=(4.5\pm0.67)\times10^5$ copies per μL. Compared the test with conventional real-time quantitative PCR (Fig. 4C), the result demonstrated the robustness of the dPCR chip (R^2=0.996). The raw statistical data are included in the electronic supplemental information (table 1).



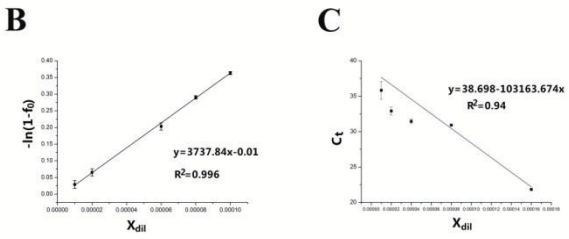


Fig. 4. Digital PCR results on the lamina-chip with different concentrations of DNA template. Caption(A): Digital PCR fluorescent imagines with a serial dilution of target DNA template. (B): A regression curve of CK19 was acquired by plotting the observed positive points in dPCR against the dilution factor x_{dil}. (C): A regression curve of CK19 was acquired by plotting the Ct value in qPCR against the dilution factor x_{dil}.

Conclusions

The microfluidic dPCR platform described here has several advantages. Sample loading is assisted by a localized temporary on-chip vacuum in channels directly adjacent to the chambers is efficient and fast. Just with the help of syringe to afford negative pressure, sample solution is “pulled” or “sucked” into every

chamber. This is an interesting way of partially controlling sample loading without valves. So if we could control the time and area of temporary on-chip vacuum, we could control the order of reagents loaded in a chamber and the area of reagents in a chip.

And the parts of chip were designed according to the functions of the modules, which were easy to be fabricated without multiple overlay exposure or high alignment precision. And the chip is user-friendly that the fabricated PDMS layers just paste on each other without complex processing to realize molecular bonding. The functions modularity makes the chip compatible with some other functional device such as DNA isolation or single cell capture.

It is difficult to prison drops of PCR mix solution in chambers under thermalcycling. It makes even worse that the evaporation of solution can permeate into PDMS to cause the imbalance of PCR solution mix. So it is an essential element for dPCR plateform to equally divide PCR solution, tightly seal the solution and restrain evaporation or moisturize.

In our dPCR platform, we successfully utilized the hydrophobicity of PDMS to divide the solution into each chamber, press the chip tightly to seal the solution by the hotlid of PCR device. We oppositely utilized the gas-permeability of PDMS to add a vaporproof-layer to restrain evaporation that caused by the gas-permeability of PDMS.

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Notes and references

[a] *Research Center for Analytical Instrumentation, Institute of CyberSystems and Control, State Key Laboratory of Industrial Control Technology, Zhejiang University, Hangzhou 310058, Zhejiang, P. R. China.*

[b] *College of Life Sciences, Zhejiang University, Hangzhou 310058, Zhejiang, P. R. China.*

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