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A Pipeline-Based Oil-Bath PCR Method for Bacteria Detection

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ABSTRACT Polymerase chain reaction (PCR) technology is an essential technology for point-of-care testing (POCT) application. To realize the simple, portable and fast detection of bacteria has always been an important technology. In this paper, we developed a pipeline-based oil-bath PCR method, to achieve the PCR amplification and detection for bacteria. We assembled a small-sized system with a uniform temperature environment and a stable heating module. By comparing the PCR performance with the other two existing pipeline-based methods on the reactions of both DNA fragment of H7N9 avian influenza and Escherichia Coli, the results show that the pipeline-based oil-bath PCR method is fully capable of the amplification and detection of both DNA fragment and bacteria. At the meanwhile, we performed two sets of tests, the experiment of pipeline diameter change and bacterial concentration gradient, to verify the sensitivity and universality of this method. The results show that the pipeline-based oil-bath PCR method developed by us not only gets rid of the complicated preparations, offers advantages of simple operation, low-cost and user-friendly, but also provides a stable and portable foundation for our later work.

INDEX TERMS Bacteria detection, oil-bath PCR method, pipeline-based.

I. INTRODUCTION

Point-of-care testing (POCT) is a new method in recent years which is to analyze the samples immediately at the sampling site and can get the test results quickly. It can eliminate the complex processing procedures when the samples are tested in the laboratory [1]–[3].

Developed in 1983 by Kary Mullis, polymerase chain reaction (PCR) technology has been known as the greatest and most widely used inventions in medical and biological researches [4]–[7]. It can simulate the replication process of natural DNA molecules, amplify specific DNA (or RNA) molecular fragments in vitro, to amplify the original target gene lower than the detection line to the level that can be analyzed for qualitative analysis [8], [9]. This technique is easy to operate and highly sensitive. It has been widely used in the detection of various pathogenic microorganisms and parasites [10]. Especially with the advent of PCR chip technology, it plays an important role in POCT [11]–[13].

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In recent years, it is more and more important to use PCR technology to realize POCT application in outfield, such as disaster rescue [14]–[16], quarantine [10], [17] and plant disease monitoring [18], [19]. Our team has recently successfully developed a portable PCR platform with low energy consumption, light weight and small size, which can be applied to the accurate analysis of a variety of nucleic acids, to achieve the characteristics of small-scale portability, simple operation, and instant report [20]. However, the sample used in the analysis is not the intact bacteria obtained from field sampling, but the sample after DNA extraction. In the past few decades, it has been a hot spot to directly analyze intact bacterial by PCR. For example, in the article "Field detection of Schistosoma japonicum cercariae in environmental water samples by quantitative PCR." published by Worrell *et al.* [21], "An integrated all foil based micro device for point of care diagnostic applications." published by Bose *et al.* [22], and "An innovative tool for moving malaria PCR detection of parasite reservoir into the field." published by Canier *et al.* [23], they all adopted the POCT microdevice to detect DNA fragments of certain microbe. However, these studies are not directly using the original

TABLE 1. Template preparation.

Target	Sequence 5'-3'	Amplicon (bp)
H7N9 avian influenza	Fw TAC AGA CAA TCC CCG ACC GA Rv GCC AAG TGT TAG CCC CAT CC	116
Escherichia Coli	Fw AGA GTT TGA TCC TGG CTC AG Rv GWA TTA CCG CGG CKG CTG	492

intact bacteria for detection, but using in situ sample DNA extraction directly or back to the laboratory for microbial DNA extraction, and then prepare reagents to achieve PCR detection. The DNA extraction process of these methods is complex and time-consuming, so it can't realize the on-the-spot real-time detection. Therefore, how to achieve the intact bacteria amplification in PCR chips has been a problem for many years. In order to solve this problem, in this paper, we systematically analyzed a variety of pipeline-based PCR chips and their corresponding bacteria amplification results.

Since recent study shows the feasibility to lyse bacteria that is independent of the type of bacteria [24], and through the experiment and analysis of the existing methods, we designed a pipeline-based oil-bath PCR reaction method for bacteria. By combining the advantages of the previous methods, the PCR reaction method based on oil bath designed by us can realize the PCR reaction of intact bacteria quickly and efficiently. This method has the following three advantages: Firstly, this method is easy to operate, does not need to carry on the bacterial DNA extraction, directly uses the bacterial to prepare the reagent to realize the PCR reaction, greatly shortens the reaction and the detection time. Secondly, compared with other methods, the temperature of this method is stable, and the reagent is heated more evenly and reacts more fully. Thirdly, the accessories of this method can be obtained simply and easily, small size, low power consumption, which lays a good foundation for our later work.

II. MATERIALS

A. MATERIAL FOR PCR REACTION

Premix Ex Taq (HS) was purchased from TaKaRa Biotechnology (Dalian) Co., Ltd (Dalian, China). The Sterile Double Distilled water was purchased from Phygene Life Sciences Co., Ltd. (PH0727; FuZhou, China). The Bovine serum albumin (BSA) was purchased from AMEKO (AS25483, Dalian, China). The DNA fragment of H7N9 avian influenza was purchased from Genewiz (Tianjin, China). The Escherichia Coli was provided by HOOKE Instruments Ltd. The primer for all the reagents were purchased from Genewiz (Tianjin, China).

The PCR reagent of the H7N9 avian influenza was composed of 1× Premix Ex Taq (HS), Sterile Double Distilled water, 0.6 mg mL^{-1} BSA, 1 μM of forward and reverse primers, and 0.00322 ng μL^{-1} of template. The PCR reagent of the Escherichia Coli was composed of 1× Premix Ex Taq (HS), Sterile Double Distilled water, 2 mg mL^{-1} BSA, 1 μM of forward and reverse primers, and $2 \times 10^5/mm^3$ of the suspension of Escherichia coli bacteria. Each test requires 50 μL of reagent.

We culture the bacteria in lysogeny broth (LB) medium (190209, Bio-way technology Co., Ltd; Shanghai, China) overnight at 37 °C in a shaking incubator (SHZ-82A, Xinbao Instrument; Jintan, China). We measure the concentration of the bacteria using a bacteria chamber (Auvon Helber Thoma, UK), adjust the concentration to $2 \times 10^5/mm^3$ and keep it constant during each experiment.

B. MATERIAL FOR ELECTROPHORETIC ANALYSIS

Agarose powder was purchased from Sigma Company (V900510, 2%; St. Louis, Mo, USA), DL2000 DNA marker (50 × 250μl; Jialan, Beijing, China), 0.5 × TBE buffer purchased from Phygene Life Sciences Co., Ltd. (PH1755; Fuzhou, China), and Nucleic Acid GelStain purchased from KeyGEN Biotechnology (Nanjing, China) were applied to analyze the amplification results, which was detected at 302 nm with UV illuminator (ZF1-IIN, JIAPENG Co., Shanghai, China).

III. RESULTS AND DISCUSSION

A. EXISTING METHODS TEST

In order to verify the conditions required for the PCR reaction of intact bacterial, we have tested the reaction conditions required by the original bacterial reagent through the existing methods. Positive control experiments were conducted using a commercial PCR instrument (LifeECO TC-96/G/H(b)C, Bioer; Hangzhou, China) under the same thermal-cycling conditions for all targets and the high-resolution multiplexed PCRs as well. The reaction condition in the commercial PCR cycler was set with an annealing stage and denaturation stage of 40 seconds, 55°C and 40 seconds, 95°C, respectively, requiring 40 thermal cycles of amplification procedure. The commercial PCR can well realize the PCR reaction of intact bacterial by putting 20 μL of reagent into a 200 μL centrifuge bottle for temperature cycling. However, its large volume (The volume is 200 mm * 250 mm * 330 mm), high power consumption (600W), the need of more complex preliminary preparation, and cannot achieve the later stage of portable integrated multi sample programmed field microbial detection device. So here we only use the results as the reference index for performance comparison.

1) PERFORMANCE OF PIPELINE BASED IN SITU PCR INSTRUMENT

Figure 1 (a) shows a commercial in situ PCR instrument (GeneTouch TC-EA-41A, Bioer; Hangzhou, China). We tested the two kinds of reagents by this method [25]. By adding reagent into a Teflon tube (ID = 1.6 mm, OD = 2 mm)

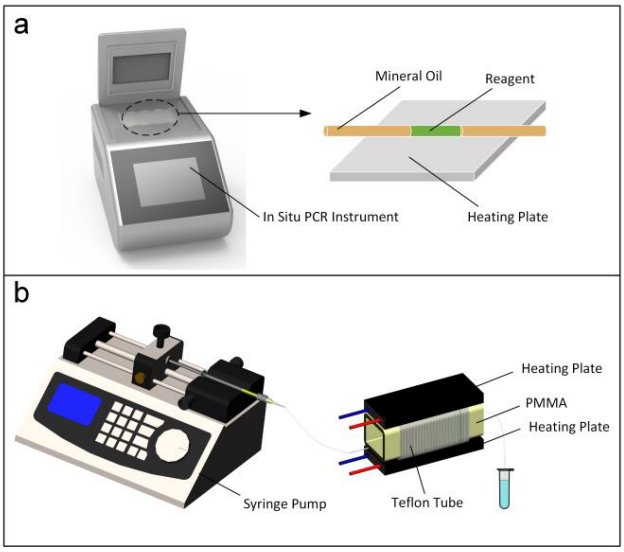

FIGURE 1. Schematic diagram of the two existing methods. (a) Pipeline based in situ PCR; (b) continuous-flow PCR.

and then fix it on the flat of the in situ PCR instrument. The design of this method is showed in Figure 1 (a). The reaction condition setting of each reagent is showed in table 2 as below.

As shown in Figure 2 (a), this method is stable and reliable with high reaction efficiency and can well achieve the amplification of H7N9 avian influenza. Theoretically, because the temperature control of the commercial in situ PCR instrument is stable and controllable, it can control the temperature cycle nicely, which should be able to realize the PCR reaction for intact bacteria. However, as shown in Figure 2 (b), there are barely no amplification products, indicating that the intact bacteria amplification efficiency of this method is extremely low. Because the cylindrical Teflon tube is attached to the surface of the flat, the heating surface is small, the heating of the reagent is uneven. The temperature close to the flat is stable, but the farther away from the heating surface, the lower the temperature. So, the PCR reaction for intact bacteria cannot be realized efficiently by this method.

2) PERFORMANCE OF CONTINUOUS-FLOW PCR SYSTEM

As shown in Figure 1 (b), based on former works, by setting up a continuous-flow PCR amplification system with double heating plates, to verify the ability of this method for achieving PCR reaction with intact bacteria [26], [27]. Before the experiment, set up the speed of the syringe pump to be 0.8 mL/h, to make sure it can achieve 40s of denaturation stage, and 40s of annealing stage. One of the two heating plates is set at 95°C and the other is set at 55°C. Before the injection of the reagent of Escherichia Coli, it should be put into commercial PCR instrument for wall breaking, the wall breaking time is 300s and the temperature is 95 °C.

As shown in Figure 2 (c), this method can also achieve PCR amplification of H7N9 avian influenza, but it is difficult to carry out PCR reaction of Escherichia Coli. Because the continuous-flow pipeline is thin, the space for the thermal movement of molecules in reagents is narrow, and the bacteria cannot fully combine with the reaction reagent in the pipeline, so the amplification efficiency is low for PCR reaction for intact bacteria

TABLE 2. PCR reaction conditions.

Reagent	Temperature (°C) / Time (s)			Cycle number
	Wall-breaking stage	Denaturation stage	Annealing stage	
H7N9 avian influenza	-	95 / 40	55 / 40	40
Escherichia Coli	95 / 300	95 / 40	55 / 40	40

B. NOVEL PIPELINE-BASED PCR METHOD FOR INTACT BACTERIA DETECTION

1) METHOD EXPLORATION

Through the former two sets of experiments, we found out that to achieve PCR reaction for intact bacteria, it needs sufficient reaction of reagents, stable temperature circulation and even heating. Based on the above requirements, we designed a new pipeline-based PCR method. First, a thicker pipeline can enhance the thermal movement of internal molecules and make the reagent fully react. Next, the heating method is simpler and more portable, and only one Thermoelectric Cooler (TEC) can realize the temperature cycle required for PCR reaction.

As we can see in Figure 3 (a), the thermal cycle module contains a TEC (TEC2-25416; Fudianzi Tech, Hebei, China), a USB fan (CJY, China), a temperature controller (TCM-M207; Sichuan, China), a bracket, a heat sink (Miaoxin, Shenzhen, China), a platinum resistor temperature sensor which is fixed by a clip (PT1000; Fengchengzekai, Beijing, China) and a script that we wrote for the automatic temperature control. The control script is coded in Python and can be written with basic programming knowledge. So, we set up the thermocycling in the script to initiate a PCR reaction which includes 40s of denaturation stage (95 °C) and 40s of annealing stage (60 °C) for 40 cycles. Then, in order to achieve more uniform heating, we adopt the method of oil bath. Low viscosity dimethyl silicone oil (100cs; Lichen Tech, Shanghai, China) has good heat resistance and thermal stability, by adding a proper amount (approximately 6-8 mL) of dimethyl silicone oil into the aluminum reaction tank, the pipeline immersed in the dimethyl silicone oil can be fully and evenly heated, and thus, the PCR reaction will be more adequate and stable. Figure 3 (b) and (c) took above the reaction tank show the infrared thermogram of the oil in the high and low temperature regions during the PCR cycle, we used an infrared (IR) camera (Fotric 220, ZXF Laboratory, Allen, TX, USA) to monitor the temperature. And as we can

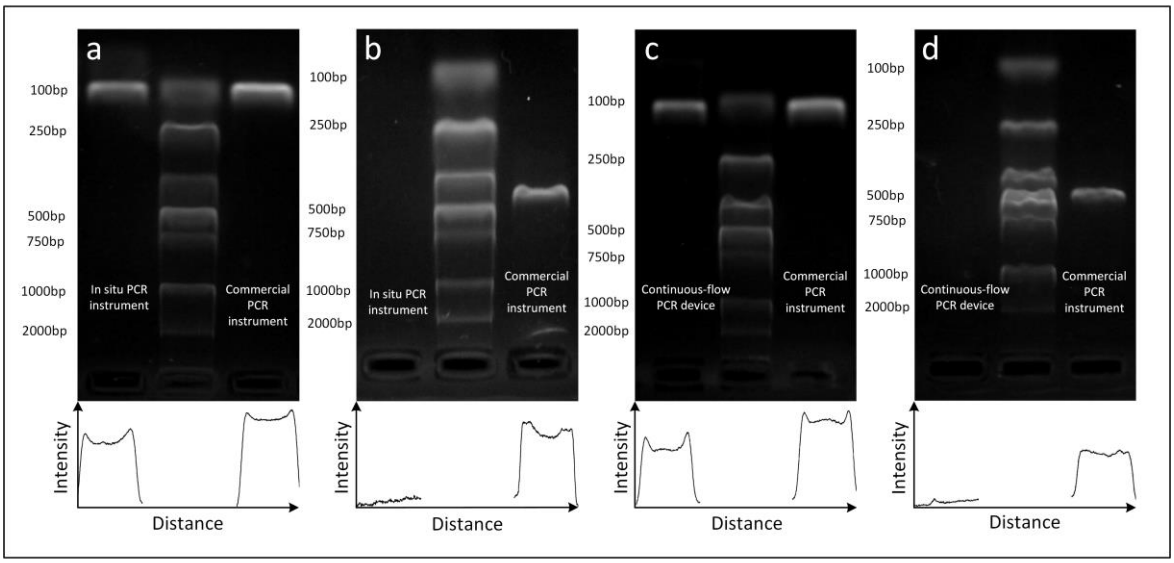

FIGURE 2. Electrophoretic results and the intensity diagram of the SDS-PAGE image by the two existing methods. (a) The amplification of H7N9 avian influenza by in situ PCR instrument; (b) The amplification of Escherichia Coli by in situ PCR instrument; (c) The amplification of H7N9 avian influenza by continuous-flow PCR device; (d) The amplification of Escherichia Coli by continuous-flow PCR device.

see in the figures, The temperature of the oil is uniform, and the temperature of each part is within the threshold range of PCR amplification, so the PCR amplification process can be well realized.

The assembly of this system is shown in figure 4 (a), the reaction tank is made of aluminum foil, and the size of it is 50 mm *50 mm *20 mm. The Teflon tube (ID = 0.8mm, OD = 1.6mm) is fixed at the bottom of the reaction tank, then put a platinum resistor temperature sensor next to the tube and fixed by a clip to monitor the temperature of the oil. Put the reaction tank on the TEC, and fix the TEC on the heat sink with the USB cooling fan beneath it. During the experiment, first, fill the Teflon tube with mineral oil (M8410-1L, SIGMA-ALDRICH; MO, USA), then inject the reagent into the middle of the tube by a pump (LANDE, LD-P2020; China) to ensure that the section containing the reagent is completely immersed in the dimethyl silicone oil, and then stop the injecting to make sure the part that contains the reagent is fixed in the oil. Then use the temperature controller to control the TEC to realize the proper temperature cycle for the PCR reaction.

Figure 4 (b) and (c) show the electrophoretic results and the intensity diagram of the SDS-PAGE image of our new PCR method. As we can see in the figure, this novel method can not only achieve the amplification of DNA fragments like H7N9 avian influenza with a high amplification efficiency, but also realize the detection and amplification of intact bacteria detection like Escherichia Coli, the amplification efficiency is nearly 75% of that of the commercial PCR instrument, which indicates that our method is fully capable of realizing PCR reaction for bacteria. Besides, the size of our microsystem is approximately 150 mm *120 mm *120 mm without the pump and power source, which is way much

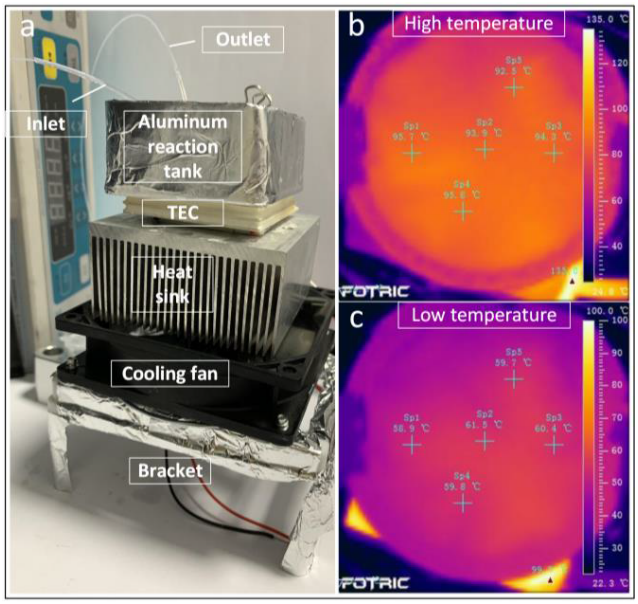

FIGURE 3. (a) The physical diagram of the system. (b) The infrared image of oil surface at high temperature; (c) The infrared image of oil surface at low temperature.

smaller than that of the commercial PCR instrument, so it can be portable enough to realize real-time detection in outfield. And the total power consumption of this system is only 220W, while commercial PCR instruments are generally 600W or above, therefore, our microsystem has the advantages of low power consumption, small volume, and uniform temperature, which can completely realize the portable and stable POCT detection.

To test the sensitivity and universality of the method, we designed two sets of experiments, the results are showed as below.

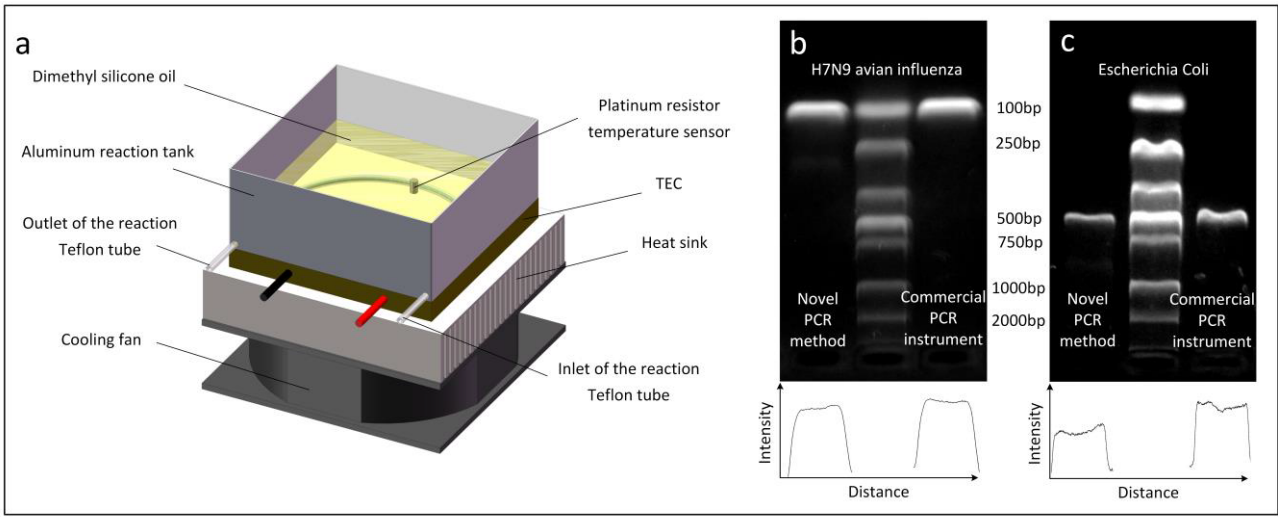

FIGURE 4. (a) The assembly of our novel pipeline-based PCR method for intact bacteria detection. And Electrophoretic results and the intensity diagram of the SDS-PAGE image by our novel PCR method. (b) The amplification of H7N9 avian influenza; (c) The amplification of Escherichia Coli.

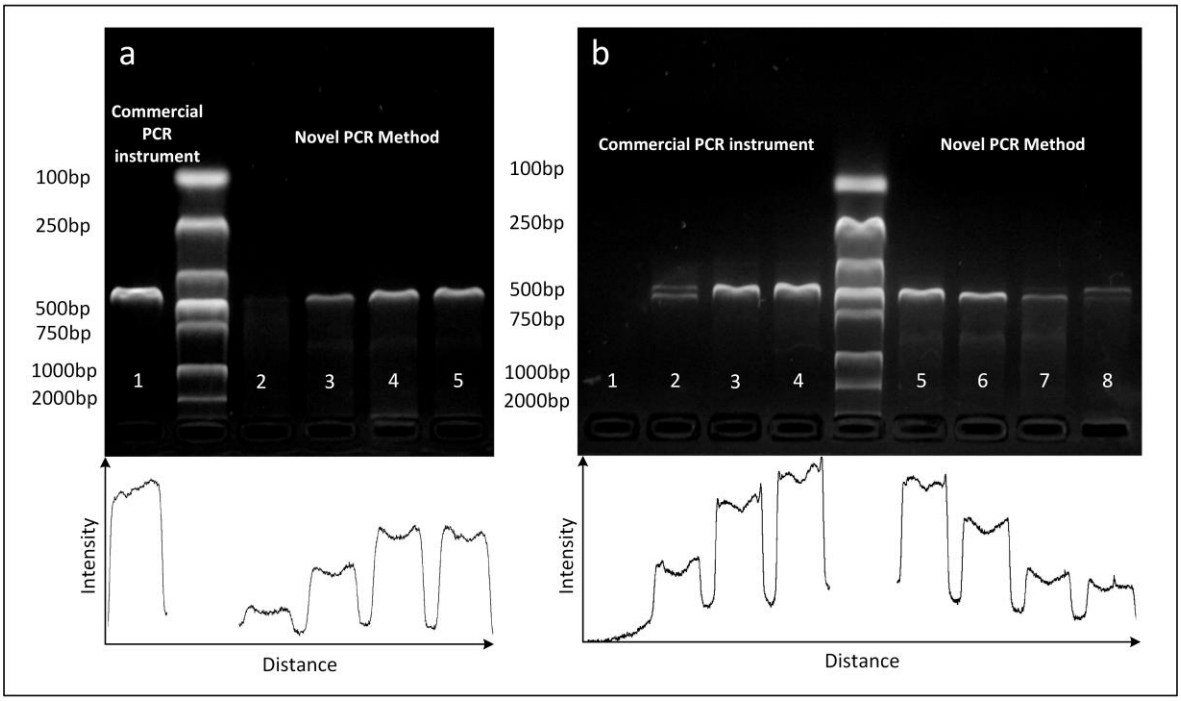

FIGURE 5. (a) Electrophoretic results and the intensity diagram of the SDS-PAGE image of experiment on changing the diameter of the Teflon tube. Lane 1 is the result of commercial PCR instrument. Lane 2 to lane 5 (b) Electrophoretic results and the intensity diagram of the SDS-PAGE image of experiment on bacterial concentration gradient. Lane 1 to 4 are the results of commercial PCR instrument. Lane 5 to lane 8 are the results of different bacterial concentration.

2) EXPERIMENT ON CHANGING THE DIAMETER OF THE TEFLON TUBE

This novel method has a good experimental flexibility, and can be easily used for experiments with different diameters of reaction pipelines. In order to verify the effect of different pipeline diameters on the experimental results, we designed a set of experiments. In this experiment, four different pipeline diameters of Teflon tubes were selected: 0.3 mm * 0.6 mm, 0.5 mm * 0.9 mm, 0.8 mm * 1.6 mm, and 1.5 mm * 2.5 mm, all of which were 20 cm long. The experimental method is the same as before.

Figure 5 (a) shows the electrophoretic results and the intensity diagram of the SDS-PAGE image of this experiment. Lane 1 is the result of commercial PCR instrument. Lane 2 to 5 are the results of different pipeline diameters, lane 2 is the result of the 0.3 mm * 0.6 mm Teflon tube, lane 3 is the result of the 0.5 mm * 0.9 mm Teflon tube, lane 4 is the result of the 0.8 mm * 1.6 mm Teflon tube, lane 5 is the result of the 1.5 mm * 2.5 mm Teflon tube.

As we can see in the figure, under such experimental conditions, the smaller the inner diameter of the Teflon tube, the lower the reaction efficiency. Because the pipeline is

thin, the space for the thermal movement of molecules inside the pipeline is narrow which cause the bacteria cannot fully combine with the reaction reagent.

3) EXPERIMENT ON BACTERIAL CONCENTRATION GRADIENT

In order to verify the reaction efficiency of this method to different concentrations of Escherichia Coli bacterial solution, we prepared four different concentrations of bacterial solution: $2 \times 10^5/\text{mm}^3$, $2 \times 10^4/\text{mm}^3$, $2 \times 10^3/\text{mm}^3$, and $2 \times 10^2/\text{mm}^3$. The reaction Teflon tube is 0.8 mm * 1.6 mm and it is 20 cm long. The reaction conditions are the same as before. The PCR results both in the commercial PCR instrument and the novel PCR device of the concentration of $2 \times 10^5/\text{mm}^3$ which we used in our earlier experiments are showed in Figure 5 (b) lane 4 and 5. The PCR results of the concentration of $2 \times 10^4/\text{mm}^3$ are showed in Figure 4 (b) lane 3 and 6. The PCR results of the concentration of $2 \times 10^3/\text{mm}^3$ are showed in Figure 5 (b) lane 2 and 7. The PCR results of the concentration of $2 \times 10^2/\text{mm}^3$ are showed in Figure 5 (b) lane 1 and 8.

As we can see in the electrophoretic results and the intensity diagram of the SDS-PAGE image of Figure 5 (b), the lower the concentration of bacterial solution, the lower the reaction efficiency. In addition, the reaction results of our microsystem to different concentrations of bacterial solution are similar to those of commercial PCR instruments.

IV. CONCLUSION

In this study, a new pipeline-based PCR method for intact bacteria was proposed. Through the experiment of the existing methods, we designed a microsystem with thicker pipeline to make the reagent react more fully, simpler and more portable heating method to achieve a more stable and controllable temperature cycle, and more uniform heating to make the reagent more evenly heated. The experimental results show that the reaction efficiency of this method is higher than that of the previous methods, and is similar to that of commercial PCR instrument. Moreover, the accessories needed by this method are easy to obtain, small in size and low in power consumption, which can be applied to our next portable integrated detection device for field detection. We will further improve the system and add reverse transcription function, so that it can be applied to the detection of COVID-19. As a technology driven product, POCT should develop towards automation, informatization and intelligent technology platform. In the future, we will further improve this method to make its performance more stable and use more convenient, so it can be perfectly applied in the field portable integrated POCT detection device.

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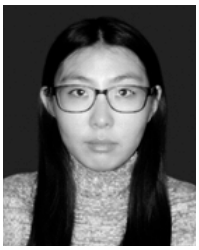

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