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Nested PCR in magnetically actuated circular closed-loop PCR microchip system

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Abstract

We demonstrate a new and sensitive amplification technique (referred to as Nested Polymerase Chain Reaction; nPCR). It based on a magnetically actuated circular closed-loop PCR microchip system. nPCR involves the use of two sets of primers in two successive PCR runs, and allows the amplification of a single locus from a minute quantity of template DNA. Two sets of primers are specially designed to a target 500-bp region of the bacteriophage lambda template DNA in the first PCR run, and a 247-bp region of the targeted 500-bp first PCR product in the second PCR run. PCR is run on the microchip system and concurrently in regular thermocycler for comparison. The products are analyzed by conventional agarose gel electrophoresis. The detection limit for the initial template DNA is 1.63×10^5 copies per μL (or 8.67 pg) for the first PCR run, and 1.63 copies per μL (or 0.0867 fg) for the second run. The results are comparable to a regular thermocycler. This preliminary study opens a new gateway to future development of specialized nPCR on chip.

Keywords Nested PCR · Magnetically actuated · PCR microchip · Lab-on-a-chip · Micro total analysis system

Introduction

Nested polymerase chain reaction (nPCR) is a powerful variant of the PCR technique, which can be used to amplify extreme low quantities of template DNA. It differs from the standard PCR protocol, mainly by the conceptual primer design, which involves two sets of primers (external and internal) that are used in two successive PCR amplification runs. The first set of external primers is designed to prime out a generic region of interest and the second set of internal primers is intended to amplify the secondary target within the region of the first run DNA amplicon. This primer design concept forms the basis for Consensus PCR [1] and Tandem PCR [2]. This nPCR can also be conducted in conjunction with reverse transcription PCR and real-time PCR. Nested PCR is more sensitive and specific as compared to a standard PCR protocol. For instance, in the case of identification of transmission sites of schistosomiasis, the limits of detection (LOD) of the parasite's DNA, *Schistosoma mansoni*, for the conventional PCR, nPCR and single-tube nPCR are 10 pg, 0.1 fg and 1 fg, respectively [3]. In the detection of *Plasmodium falciparum* for Malaria, six parasites per μL can be detected with nPCR [4]. The sensitivity of Ross River virus detection can be up to 0.1 per mL plaque forming unit [5]. The two sets of primers have to be exactly complementary to the target DNA in order to have positive amplification. Thus, it can be used to reduce the common issue of unintended product, when detecting a particular strain of bacteria from a mixture of microbial flora and patient's genome. As the PCR runs are carried out twice, large quantities of PCR products are produced. This is especially useful for pathogen detection or forensics, where a limited amount of nucleic acid template is provided. Nested PCR is useful to identify serotypes of virus [6–10], parasites [3, 4] and bacteria [11].

PCR microchips are specialised lab-on-chip (LOC) that uses PCR and its variants for the detection of pathogens, biomarkers and DNA fingerprinting [12, 13]. PCR on chip system can be implemented in the form of well-based or continuous-flow [13]. In continuous-flow PCR system, it could be further sub-categorized to closed-loop [14], fixed-loop [15] and oscillatory [16]. Although the first PCR on chip was introduced by Northrup *et al.* in 1993 [17], nested PCR protocol was rarely implemented in LOC related literatures. Most of the previous works demonstrated the use of nPCR amplification in regular thermocyclers with DNA fragment analysis on a LOC system, hence these works cannot be considered truly as nPCR on chip.

Focke *et al.* had recently introduced a new centrifugal microfluidic system designed for primary amplification and secondary real-time PCR [18]. However, in their work, the first PCR run was done on the microchip system. Subsequently, the amplified product was aliquot again for real-time PCR in a modified commercial thermocycler. Hence, at the end, only half of the protocol was done on chip. Sciancalepore *et al.* demonstrated nested PCR for tyrosinase gene detection on their oscillatory flow microchip system [16], in which the PCR mixture was embedded in oil phase, moved back and forth, oscillating through three temperature zone. Successful PCR runs were achieved by passing this microchip for first PCR run and then again for the second PCR run. Although nPCR is one of the well-known PCRs and an extremely powerful technique in molecular cell biology (MCB), currently there is only one article that has successfully implemented the complete nPCR protocol and is reported in the field of LOC.

In our research group, Sun *et al.* had developed the circular closed-loop PCR microchip system that used magnetic actuation to propel the PCR mixture along the micro-channel through different temperature zones [14]. This system offered the flexibility in the number of PCR cycles and this concept was developed further to provide high-throughput by creating parallel closed-loop circular channels [19]. This microchip system had been used for DNA analysis in genetically modified organisms, in which multiplex DNA amplification was indirectly demonstrated [20]. The design of the microchip was further modified to allow sample loading, using a benchtop centrifuge [21]. Although this PCR system had been reported several years ago, it had never been demonstrated for other PCR variants other than the previous truncated PCR protocol [14].

Here, we reported the first implementation of nested PCR protocol in closed-loop PCR on chip system. The complete nested PCR protocol (first and second PCR run) was demonstrated in our system [14], using the DNA amplification in bacteriophage lambda DNA as a model. Two sets of primers were designed to prime out a region of interest. Nested PCR was carried out to amplify this selected region of interest at lowering concentrations of the template DNA. The detection limits were determined. Results showed a promising potential for future development of specialised integrated nPCR-CE LOC. Therefore, our current study provides the preliminary knowledge for implementing nested PCR protocol on LOC system. Hereby, we highlight the potential of implementing this complicated protocol in microfluidic devices,

in hope for a better development of specialised nested PCR LOC devices for MCB laboratory work.

Experimental

Microchip system design

Ester oil-based ferrofluid and PCR mixture were loaded onto the access port of the microchip using a pipette. This microchip was then inserted into a 50-mL centrifuge tube and centrifuged at 3000 rpm/1730×g for three minutes, in order to load the sample into the circular channel [21]. The chip was placed then onto the microchip PCR system for PCR amplification. Figure 1 features the overall schematic arrangement of this microchip PCR system. The heater unit consisted of three blocks which provided the required temperature for denaturation, annealing and extension steps. The external rotary permanent magnet actuated the dynamic magnetic plug in the microchip [14, 19]. Since the oil-based plug was immiscible with the aqueous PCR mixture medium, the mixture was propelled by the movement of this plug along the channel through the various discrete temperature zones. One thermal cycle in this system was 36 s.

Nested PCR model

Bacteriophage lambda DNA was chosen as the template DNA because it was available commercially. The molecular weight of the DNA strand used was 3.2×10^7 g.mol⁻¹ or 5.31×10^{-17} g. Using standard nested PCR protocol, two sets of oligonucleotide primers (external and internal) were used in this study. These primers were synthesized by and purchased from 1st BASE (Singapore, www.base-asia.com). The set of external primers was designed to amplify a 500 bp of DNA product from the template DNA. The oligonucleotide primer sequence was adopted from Sun *et al.*'s work [14]. The internal primers set were designed to amplify 247 bp of DNA product within the 500-bp template DNA. This oligonucleotide sequence was selected using primer-Blast. Table 1 lists the detailed information about the primers used.

For each experiment conducted, the final PCR mixture composed of 1× goTaq© Hot Start Green Master Mix, 50 nM of forward primers and reverse primers, 1 µg.µL⁻¹ of Bovine Serum Albumin (BSA) and varied amounts of template DNA. This mixture was topped up with

nuclease-free water. All reagents were obtained from Promega (USA, www.promega.com). The stock lambda DNA had a concentration of $867 \text{ ng} \cdot \mu\text{L}^{-1}$ or $1.63 \times 10^{10} \text{ copies} \cdot \mu\text{L}^{-1}$. It was further diluted for use. A non-template control, N, was introduced as a negative control; equivalent amount of nuclease-free water was used in replacement of the diluted template.

nPCR protocol in microchip system

In the first PCR run, an amount of $0.5 \mu\text{L}$ of the diluted template DNA was added to $4.5 \mu\text{L}$ of the PCR master mixture, containing the external primers. $2 \mu\text{L}$ of ferrofluid and $5 \mu\text{L}$ of PCR mixture were loaded onto the access port of the circular closed-loop PCR microchip and centrifuged at $3000 \text{ rpm}/1730 \times g$ for 3 min. Silicon or mineral oil was used to fill up the access port of this microchip, to prevent the evaporation of the samples. The loaded microchip was then placed into the microchip system. PCR was carried out as explained earlier. At first, all heater blocks were set at 95°C for 2 min, to inactivate the antibody and initiate the hot-start PCR. Subsequently, the heaters were set to 60°C , 72°C and 95°C , respectively as shown in Fig. 1. The external rotary magnet was switched on for 15 min, which was equivalent to 25 thermal cycles. PCR was carried out when the PCR mixture moved through the different temperature zones. Finally the blocks were set at 72°C for 3 min. The total runtime was 20 min. The DNA products were retrieved by centrifuging the inverted microchip into a clean sterile 50-mL centrifuge tube, using a regular benchtop centrifuge at $3000 \text{ rpm}/1730 \times g$ for 3 min.

After the first PCR run, $0.5 \mu\text{L}$ of the run DNA product was subsequently added into the microchip which contained $4.5 \mu\text{L}$ of the PCR master mixture for the second PCR run. This new PCR master mixture contained the internal primers. The second PCR run was carried out similarly as described in the first PCR run.

To find out the lowest detectable initial template DNA concentration that can be established in the microchip system, a serial dilution was carried out with nuclease-free water. The final concentration of the template DNA was ranged from 1.63×10^{-1} to $1.63 \times 10^9 \text{ copies} \cdot \mu\text{L}^{-1}$.

Comparison with thermocycler

To compare between the microchip system and the conventional thermocycler system, nested PCR was also conducted in a regular thermocycler. The thermocycler, GeneAmp PCR system 9700, was purchased from Life Technologies (USA, www.lifetechnologies.com). A similar nested PCR protocol was used. For the first PCR run, 10 μ L or 20 μ L of the PCR mixtures were amplified in 0.2-mL Eppendorf tubes. A PCR thermal cycling protocol was setup as such: denaturation at 95 °C for three minutes, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, and finally an extended extension step at 72 °C for 3 min. The run product was kept at 25 °C. For the second PCR run, the previous DNA product was added into the new PCR mixture (1:10). The similar thermal cycling protocol was used. The total runtime for the thermocycler system for the nested PCR protocol was 2 h and 24 min.

DNA analysis

All PCR processed samples from PCR runs were analysed with 0.8% agarose stack gel electrophoresis with gelREDTM using a 1× TBE buffer. GelRedTM was obtained from Biotium (CA, USA, www.biotium.com), and it served as a replacement for ethidium bromide staining. A sample amount of approximately 5 μ L from the PCR runs was loaded onto the gel for electrophoresis at 80 mV for 45 min. The gel was then read using a UV transilluminator. DNA band sizes were determined relative to a 1 kb DNA ladder. The presence of a 247-bp and 500-bp DNA band was recorded as a positive result. The images were further analysed using Gel Analyzer, gel image analysis software that was downloaded from gelanalyzer.com, to obtain the raw volume and peak intensity value of the DNA band. The values were normalized against the background and the amount of sample loaded. The outliers were discarded and the collective data of three to five repeated experiments were compiled.

Results and discussion

Primers optimization

The concentration of the primers used in the PCR reaction affects the DNA amplification result dramatically. A multiplex PCR reaction may occur, when both external and internal primers are presented in the PCR mixture at a high concentration. To investigate the optimized concentration of primers to be used in the nPCR, varying amounts of primers from 50 nM to 1 μ M was evaluated. PCR conditions and DNA analysis were as stated earlier. Gel electrophoresis was chosen as the analytical method because downstream protocol, such as gel extraction may follow for further analysis. DNA sequencing may be carried out to further identify the genotype of the pathogen.

When both sets of internal and external primers were presented in a high concentration of 1 μ M each in the PCR mixture at an annealing temperature of 60 °C, with sufficient template DNA, exactly four DNA bands could be detected, Fig. 2. The external and internal primers can inter-pair together resulting in products of 247-bp, 324-bp, 423-bp and 500-bp. These are expected products, predicted by using primer-blast. This inter-primer pairing produced a unique amplicon signature of this lambda DNA, which may be used for pathogen identification. However, to simplify the DNA analysis in this work, clear bands of 247-bp and 500-bp were preferred.

The concentrations of the primers were lowered from 1 μ M to 50 nM in the PCR mixture. When both primer sets were used at low concentrations, clear bands of 247-bp and 500-bp were observed in the gel image. The concentration of primer was finally optimized at 50 nM in the final PCR mixture. This is lower than the recommended final concentration of 0.1 μ M of primer recommended in the manufacturer's manual. The issue of reducing multiplex reaction can also be resolved by lowering the concentration of the external primers in the first reaction. An alternative would be optimizing the annealing temperature of the primers. This notion is important for the future development of specialised integrated nPCR microchip.

Nested PCR

Nested PCR was conducted on the magnetically actuated circular closed-loop PCR microchip system. $86.7 \text{ ng.}\mu\text{L}^{-1}$ or $1.63 \times 10^9 \text{ copies.}\mu\text{L}^{-1}$ of template lambda DNA was added to the PCR mixture. PCR conditions were stated earlier and the products were analysed using gel electrophoresis. In the first PCR run, the external primers were used to amplify the 500- bp DNA product from the minute amount of lambda template DNA. In the second PCR run, the internal primers were used to amplify the 247-bp DNA product from the 500-bp amplified template DNA from the first PCR run. The results showed that both bands of 500-bp and 247-bp could be detected in the first and second PCR run respectively.

To investigate the detection limit of the PCR system, a series of ten-fold dilutions (1.63×10^{-1} to $1.63 \times 10^9 \text{ copies.}\mu\text{L}^{-1}$) were carried out on the template DNA. Subsequently nested PCR was conducted with these concentrations of diluted template DNA. With adequate template DNA in the PCR mixture, PCR would be able to proceed successfully, producing DNA bands that would be observable in the gel under UV light, after gel electrophoresis.

Figure 3 shows the UV image of the PCR products obtained after gel electrophoresis. The relative sizes of the band were measured by comparing to the 1kB ladder. In the first PCR run, 500-bp bands were observed from 1.63×10^5 to $1.63 \times 10^9 \text{ copies.}\mu\text{L}^{-1}$ for both PCR systems. In the second PCR run, 247-bp bands were observed from 1.63×10^1 to $1.63 \times 10^9 \text{ copies.}\mu\text{L}^{-1}$ for both PCR systems. Controls without the DNA template showed no bands. The last visible 247-bp band was observed at the concentration of $16.3 \text{ copies.}\mu\text{L}^{-1}$. As observed from the gel image in Fig. 3, the first PCR run may not show a 500-bp positive band for initial template DNA of 16.3 to $1.63 \times 10^4 \text{ copies.}\mu\text{L}^{-1}$, even though this 500-bp DNA product was presented, but in very low concentrations. If there were no 500-bp product in these tests, there would be no positive 247-bp band obtained in the second PCR run. The second PCR run uses internal primers that target this 500-bp amplicon to further amplify a 247-bp portion of it. This shows that nested PCR can be used to detect the presence of low quantities of template DNA.

To provide more information about the band products from the UV image, the image was imported to gel analyzer to extract the intensities of the bands. Figure 4 shows the initial DNA template concentrations against the DNA product bands intensities in the PCR systems. In the first PCR run, for both PCR systems, increasing band intensity was observed starting from

1.63×10^5 to 1.63×10^7 copies. μL^{-1} . Band intensity showed maximum at 1.63×10^7 copies. μL^{-1} , and decreased after 1.63×10^7 copies. μL^{-1} . The detection limit was 1.63×10^5 copies. μL^{-1} (or 8.67 pg). In Melo *et al.*'s work, the limit of detection (LOD) for the nPCR in detecting parasite's DNA was 10 pg [3], which was comparable to our result of 8.67 pg. For the second PCR run, band intensities generally increased from 1.63 to 1.63×10^9 copies. μL^{-1} . For the microchip system, there was an incline in the band intensities, whereas, for the thermocycler system, from 1.63×10^4 to 1.63×10^9 copies. μL^{-1} , the band intensities start to plateau.

Since the band intensities for 1.63 copies. μL^{-1} of template DNA were 81.4 ± 6 and 89.6 ± 4 counts for the microchip and thermocycler system respectively, they were approximately three times above the background signal of ~ 15 counts. For both systems, the detection limit could be 1.63 copies. μL^{-1} or 0.0867 fg for the second PCR run. This was an extremely low quantity. A LOD of 0.0867 fg was closed to the LOD of 0.1 fg in the nPCR of Melo *et al.*'s work [3]. When the template DNA is less than 1% in the final mixture, the total number of DNA strands after n cycles is $\approx 2^n$ [22]. Assuming that PCR conditions are perfect and a single copy of template DNA is given, after two successive PCR runs of 25 cycles each, there will be an estimated 2^{50} or 1.12×10^{15} copies of DNA products. One copy of template DNA can be amplified 10^{15} times in two successive PCR runs. Hence, it is expected that extreme low quantities of DNA can be detected with nPCR.

However, it is important to note that when the experiments were repeated, nPCR with 1.63 copies. μL^{-1} of template DNA does not always produce a positive band in the microchip system. This is because the template DNA is a physical molecule, which may not be picked up by the pipette during sampling. Assuming a single copy of template DNA is required for DNA amplification, probability of picking up the single copy in a concentration of 1.63 copies. μL^{-1} is 1.63 copies in 1 μL of sampling volume. The sampling volume used in the microchip system was 0.5 μL , hence the chance of 0.8 copies of template DNA may be picked up in this volume. Therefore, there is 80% success in detecting a single copy of lambda DNA in extreme low concentrations in the microchip system. In the case of impure samples that consist of a mixture of pathogen's and patient's genome, this probability is even lower. It is recommended to mix the sample well, and do repeated experiments to confirm the results. In the thermocycler system, a larger sampling volume may be used to compensate the low con-

centration of template DNA.

On the average, the intensities of the product bands in the thermocycler system were twice and 1.4 times more compared to the microchip system for first and second PCR run respectively. The amounts of product samples were loaded equally at the volume of around 5 μL for gel electrophoresis. The intensity values of the bands had been normalized against the background and the amount being loaded into the gel. Considering these two factors, the amplification efficiency of the microchip system seemed to be inferior compared to the thermocycler system. In the case of the microchip system, a total volume of 5 μL PCR product was barely obtained after the PCR run. While in the case of thermocycler system, 5 μL of PCR product could be easily aliquoted from a total volume of 10 μL or 20 μL . Therefore, the amount of samples that were loaded into the wells of the gel for electrophoresis analysis was slightly unequal. Regardless, nested PCR was proven to be successful in our microchip system, as positive product bands can be observed using gel electrophoresis. Moreover, the detection limits were equivalent between our microchip and thermocycler system.

The advantage of performing nPCR in our microchip system is the short runtime. The total runtime for a conventional thermocycler to perform two PCR runs was about 2 h 24 min. On the other hand, the microchip system took only 40 min which is 70% less time compared to the thermocycler system. This 40-min runtime is comparable to Sciancalepore *et al.*'s microfluidic device which took 46.5 min [16]. The thermocycler system that was used in this study was considered to be an older model. Currently, modern thermocyclers may complete a general PCR in 30 to 40 min. However, two successive PCR runs will take an hour to 1 h 20 min. This duration of this runtime would further extend if the number of required PCR cycles increased. High throughput was available in the four sample closed-loop microchip as described in an earlier work [19]. This close-loop microchip system offers flexibility in the number of PCR cycles, unlike fixed-loop PCR system. This microchip system can be engineered to palm-sized. Compared to a regular thermocycler, this is a substantial reduction in bench space required. Each microchip is made of acrylic, which can be cheaply mass fabricated compared to glass, silicon or quartz. With the implementation of the microfluidic devices, molecular diagnostics approaches can be more responsive in identification of pathogens, compare to enzyme-linked immunosorbent assay or traditional cell culture, during seasonal flu or infectious outbreak [23,

24]. The integrated nested PCR microchip system may be used for personalized medicine in order to provide point-of-care test.

Our current study reported in this paper is not extensive. PCR amplification has low detection limit because the commercialized lambda DNA is highly purified. The concentration of magnesium ions can be optimized, and addition of adjuvant such as dimethyl sulfoxide (DMSO) or betaine can also improve yield for this target. For DNA fragment analysis, although agarose stack gel electrophoresis is the most common technique used in laboratories, it may not be the most sensitive. Alternative techniques such as polyacrylamide gel (PAGE) or capillary electrophoresis can provide a better resolving power in DNA separation. Hence, the lower limit of detection stated here can be lower or higher.

Putting the above disclaimers asides, this paper focused on demonstrating the proficiency of performing nested PCR using a magnetically actuated circular closed-loop PCR microchip system. Nested PCR was first successfully demonstrated in this close-loop PCR system. Although nested PCR had been demonstrated in Sciancalepore *et al.*'s oscillatory flow PCR system [16], the fundamental working mechanism was different. Hence, the future implementation of nPCR on chip will also be different.

More importantly, future development of miniaturized devices can incorporate the implementation of a specialised nested PCR microchip. Nested PCR involves two successive PCR runs. The first PCR is run using external primer. Subsequently, internal primers and reagents are added to this run PCR product in the second run. Such a complicated protocol requires the design of an intricate microchannel network, on a microchip platform to allow for accurate aliquot and amplification simultaneously. A proposed possible nPCR on chip design can be illustrated in Fig. 5. The first PCR run can amplify a generic biomarker for a particular pathogen in the top circular closed-loop. The second PCR runs can amplify the possible sub-strain biomarker for this pathogen in the four concentric lower closed-loops. A microvalve is located between the top and bottom loops to control fluidic flow. The DNA products can be analysed as a single sample. The sub-strain biomarkers may produce different DNA band size which can be differentiated by electrophoresis separation, giving a result similar to Fig. 2. The final DNA product will consist of five or more DNA bands on the gel photo.

Once a specialized nested PCR microchip is developed, it should allow for a new level of pathogen detection in miniaturized devices. Future work may also involve integrated nPCR-CE microchip. The sample can be subjected to both primary and secondary PCR, followed by its direct detection with microchip capillary electrophoresis. Manual transfer of the samples can also be omitted to reduce the chances of cross-contamination. Lastly, sensitivity of detection can be increased and analytical time can be reduced.

Conclusions

Nested PCR was first demonstrated in the magnetically actuated circular closed-loop PCR microchip system, using DNA amplification of lambda DNA as a model. The detection limit for the concentration of initial template DNA was 1.63×10^5 copies. μL^{-1} or 8.67 pg for the first PCR run, and was 1.63 copies. μL^{-1} or 0.867 fg for the second PCR run. This study provides an enlightening insight on implementing nPCR for LOC devices. Low concentration of primers needed to be used to prevent multiplex reaction. Low amount of DNA template can be detected. Although, the intensities of product band in the thermocycler system were higher than microchip system, the detection limits were equivalent. Run time for the microchip system was significantly shorter for nested PCR protocol. Integrated nPCR and nPCR-CE microchip can be devised in the future to enhance PCR on chip system.

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- Fig. 4 A graph of concentration of initial template copies in the PCR mixture against band intensity for the PCR systems. Error bars represent standard derivations derived from three to five repeated experiments. *Filled triangle* and $\times$ represent first and second PCR run in the microchip system respectively; *filled square* and *filled diamond* represent first and second PCR run in the thermocycler system respectively
- Fig. 5 A proposed specialised nested PCR on chip

Primers	DNA sequences (5'→3')		T _m , °C	GC%	Expected Product, bp
External	Forward	GATGAGTTCGTGTCCGTACAACT	60.47	47.83	500
	Reverse	GGTATCGAAATCAGCCACAGCG	66.94	52.17	
Internal	Forward	AAAGATGAACTGATTGCCCCG	60.07	45.00	247
	Reverse	CCGTGACCAGTTCAGACGTA	59.74	55.00	

Table 1

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5