PCR Micro-Volume Device for Detection of Nucleic Acids

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ABSTRACT

The polymerase chain reaction (PCR) is a nucleic acid amplifier process that is employed extensively throughout the medical and biological sciences. The technique displays an exquisite sensitivity, but an inability to distinguish trace contaminants plus high operating costs greatly reduce its impact on further potential applications. Besides low reactant consumption, a microfluidic device has attractive intrinsic properties: it can alleviate risks of contamination.

The proof of principle experiment was conducted with a modified glass capillary. PCR was performed without any optimization and the sample was then analyzed using an Agilent 2100 Bioanalyzer. The specifically amplified product was determined to have a concentration of 0.36ng/uL. Comparison with positive controls indicated that our experiment had a calculated reaction efficiency of 86% per cycle. Further experiments will be needed in order to optimize the system.

Keywords: PCR, DNA, microfluidics

1 INTRODUCTION

Despite being a key technique in clinical applications, polymerase chain reaction (PCR) presents limitations. The sequential steps leading to a successful diagnosis involve, but are not limited to, sample storage, as well as its preparation and treatment for PCR followed by data handling and interpretation. During any of these operations the operator/technician is confronted with possible sample degradation, losses and contamination. Furthermore imprecise sample tracking can lead to disastrously incorrect clinical therapy if not properly handled. We believe that within a lab-on-a-chip approach exists a solution to address these problems: if the sample is contained at all times in the chip the possible causes for contamination, mislabeling etc. are inherently limited.

Since the concept of chip-based analysis system was first introduced, the variety of applications has remarkably broadened [1, 2]. With regards to PCR, Northrup et al. used PCR chips containing reaction chambers of 25 or 50 microliters as early as 1993 [3]. The chambers were subjected to temperature cycles by means of polysilicon heaters. Kopp and co-workers developed an alternative approach in 1997 when they introduced continuous-flow PCR [4]. The sample was pumped through a meandrous channel and thermocycling was performed with the sample running over three fixed heating zones. Both methods proved quite successful but presented some disadvantages. It is for example difficult in such a device to achieve a multi-sample analysis without a significant increase in the heating design complexity or in the microfluidic chip size.

In this paper we introduce a novel approach that should present several improvements over the existing ones: with respect to the fast cycling conditions of the Kopp design it associates a greater flexibility regarding the number of cycles. Indeed the sample is shunted back and forth over two/three heating zone (see figure 1). This means that by only modifying the pumping program we can very easily increase or decrease the number of cycles with no need to manufacture a new chip. Furthermore, channel parallelisation, which allows for the basic clinical requirement of a high analysis sample throughput per unit area, can very easily be implemented based on this technique. In order to validate our model we performed several experiments. The first one consisted of a comparison of good thermocycling conditions vs. good contact conditions during an annealing/extension temperature study. Based on the results obtained, we then shunted the sample back and forth inside a glass capillary. The results of these experiments are presented in this paper.

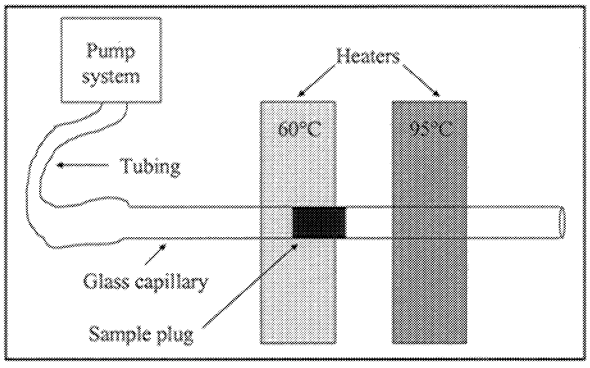

Figure 1: Schematic drawing of the shunting system. The thermocycling conditions were provided by shunting the sample plug back and forth over the heating zones (the given temperatures are only examples).

2 APPARATUS

The thermocycler used for the extension study was the Perkin Elmer 480 DNA Thermal Cycler. 0.5ml GeneAmp Reaction tubes were purchased from PE Biosystems (part number: N801-0180). The glass capillaries were LightCycler Capillaries manufactured by Roche Diagnostics GmbH (catalogue number: 1 909 339). A standard LightCycler-DNA Master SYBR Green I kit (Roche Diagnostics GmbH, catalogue number: 2 015 099) was used for the PCR reactants in association with the protooncogene MYCN Exon 2 (forward primer: GCCGAGCTGCTCCACGT; reverse primer: TCAAACTCGAGGTCTGGGTTCT).

With respect to the proof-of-principle experiment, the chemicals were identical to those previously described. The LightCycler capillaries were modified by carefully removing their ends. The homemade heating system (fig.2) consisted of three heating cartridges (20W, 120V, RS catalogue number: 837-919) inserted into three copper blocks (30mmx12mmx15mm). Pt 100 thermocouples (RS catalogue number: 362-9799) provided a feedback loop to the temperature controllers (CAL Controls, model CAL 3200). The pump used to shunt the sample was a Kloehn pump controlled by a home-written LabView program. The connection between the pump and the capillary was insured by a Microbore PTFE tube (OD: 0,042"; ID: 0.022", Cole Parmer catalogue number: EW-06417-21). This was glued to the capillary with standard Araldite epoxy.

In both sets of experiments the samples were analyzed using the Agilent 2100 Bioanalyzer in combination with the DNA 500 Assay kit (Agilent catalogue number: 5064-8284). Caution: As the dye used with the DNA 500 kit binds to DNA it should be treated as a potential mutagen and handled with care.

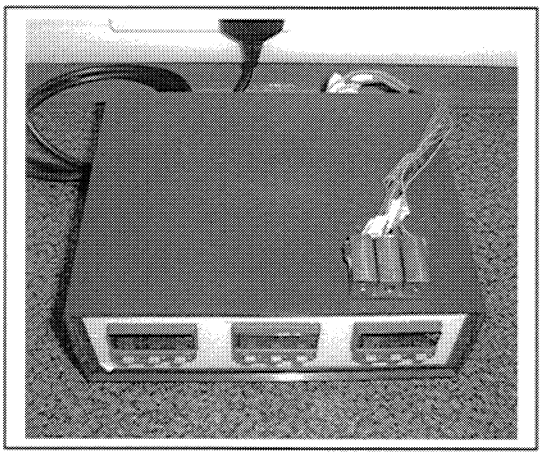

Figure 2: Home made heating device. Three heating cartridges were inserted into copper blocks. Pt100 thermocouples provide a feedback loop to the controllers

3 METHODOLOGY

3.1 Annealing/Extension temperature study

During this experiment our attention was primarily focused on the effect of good contact vs. good thermocycling conditions over the PCR reaction. As DNA strands are prone to surface adsorption, we decided to use for this experiment reactants whose adsorption-free characteristics had already been acknowledged. The commercially available LightCycler-DNA Master SYBR Green I kit presents adequate properties and the LightCycler capillaries are a valid alternative to on-chip capillaries. A master reaction was prepared as suggested by the manufacturer.

The extension temperature study was performed with several types of sample condition. The first category ensured that the instrument and the PCR were performing in a controlled and reliable way. It consisted of a DNA replication no-inhibition control (positive reagent control) and of a template-free primer control (negative control) prepared in conventional 0.5ml tubes. To avoid any sample loss due to evaporation, a few drops of mineral oil were carefully added over the PCR reactants. The second category of samples comprised capillary positive controls serving as high (100%) efficiency references. At first the PCR reactants were inserted into the capillary, subsequently centrifuged at 6000rpm for 10s to enable the sample to be at the bottom of the capillary. The capillary was finally inserted into a thermocycler well. Sufficient mineral oil was then added into the well to ensure a good thermal contact between the capillary and the sample holding block. The third and fourth sets of samples consisted of capillaries prepared exactly as the capillary positive control samples but the capillary mounting on the thermocycler differed. In one set the capillary rested on the heating block, providing good thermocycling conditions. The final capillary had its ends covered with Blue Tack^{Tm} to offer good contact. To ensure the reproducibility of the results duplicates of each sample were prepared, treated and ran identically. The PCR program was as followed: 10 min at 95°C then 40 cycles composed of one step of 15s at 95°C and another step of 60s at the studied extension temperature. The annealing/extension temperatures investigated ranged from 54°C to 64°C. The maximum heating/cooling rate of the thermocycler was 1°C/s.

3.2 Proof-of-principle study

During the proof-of-principle experiment the capillary was rested on top of two copper blocks (fig.1). The regions of the capillary in contact with the heaters were covered with Blue Tack^{Tm} (fig. 3). The temperature controller was set to maintain one heating block at 60°C and the other one at 95°C. It is to be noted that due to a poor response to the feedback loop, it was not possible to control the temperatures accurately. Independent thermocouples indicated that the respective temperatures were 68°C and 115°C. Despite all our efforts any attempt to improve the temperature control failed. The thermocycling program had to be modified accordingly so that the sample would not boil: 1min at 68°C and less than a second at 115°C. Prior to PCR the capillary was thoroughly rinsed with distilled water and then with ethanol. The sample plug was introduced in the capillary between two mineral oil plugs. This precaution was necessary in order to avoid the sample plug being diluted into the solvent. The sample plug was then shunted back and forth from one temperature zone to the other for 40 cycles.

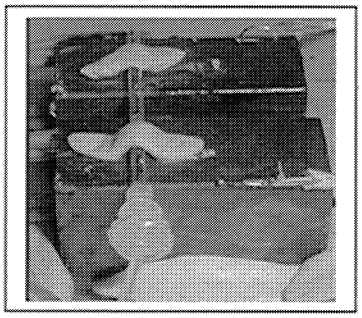

Figure 3: Proof-of-principle set-up. A glass capillary is rested on home made heating blocks.

4 RESULTS

Figure 4 shows the results of the annealing/extension temperature test, during which we studied the effect of thermal contact vs. good thermocycling conditions. We plotted the value of the fluorescence peak area determined by the Agilent 2100 Bioanalyzer against the extension/annealing temperature. As the fluorescence peak area is directly proportional to the PCR amplified products it is a good indicator of the overall efficiency. An obvious first conclusion that can be drawn when comparing the three plots is that the PCR efficiency is considerably diminished when heat transfer is impaired: a generally 40% yield difference between the reference capillary yield and the covered capillary can be noticed. This number drastically increases to 80% when comparing the reference to the uncovered capillary. Secondly the curve 2 (capillary with its end covered with Blue Tack^{Tm}) constantly displays a higher specifically amplified product yield than the curve 3. This result strongly underlines the importance of a good contact with the heaters over good thermocycling conditions. Thirdly the reference curve exhibits an increase in amplified product concentration of up to 40% when the annealing/extension temperature is optimized (60°C). It is to be noted that despite a notable loss of efficiency for the reference capillary at 64°C, a considerable increase of the specifically amplified product concentration was observed for the uncovered as well as the covered capillaries. This behavior suggests that the heat transfer loss due to poor contact is being compensated by the increase in temperature.

The electropherogram obtained with the Agilent 2100 Bioanalyzer during the proof-of-principle sample analysis can be seen in figure 5. Despite the sub-optimal thermocycling conditions depicted in the methodology section, a peak corresponding to the specifically amplified product can be detected at a migration time of 47.80s. Considering the bioanalyzer sizing resolution (± 5bp from 25-100bp), the calculated length of 54bp is in agreement with the value of 58bp obtained with the positive controls. Based on the peak area a concentration of 0.36ng/µl was determined which corresponds to an 86% efficiency per single cycle. Although the overall efficiency needs to be improved, the proof-of-principle experiment was quite successful as we could indeed detect specifically amplified products. The heating device presently used needs some modifications to be reliable but we are confident that once this is done a drastic improvement will be noted.

5 CONCLUSION

A successful series of experiments was presented in this article. It was proven that good thermal contact is crucial for an efficient polymerase chain reaction. A sample shunting approach was also demonstrated to be viable and of good potential. A more reliable heating system is required and further work will be planned accordingly.

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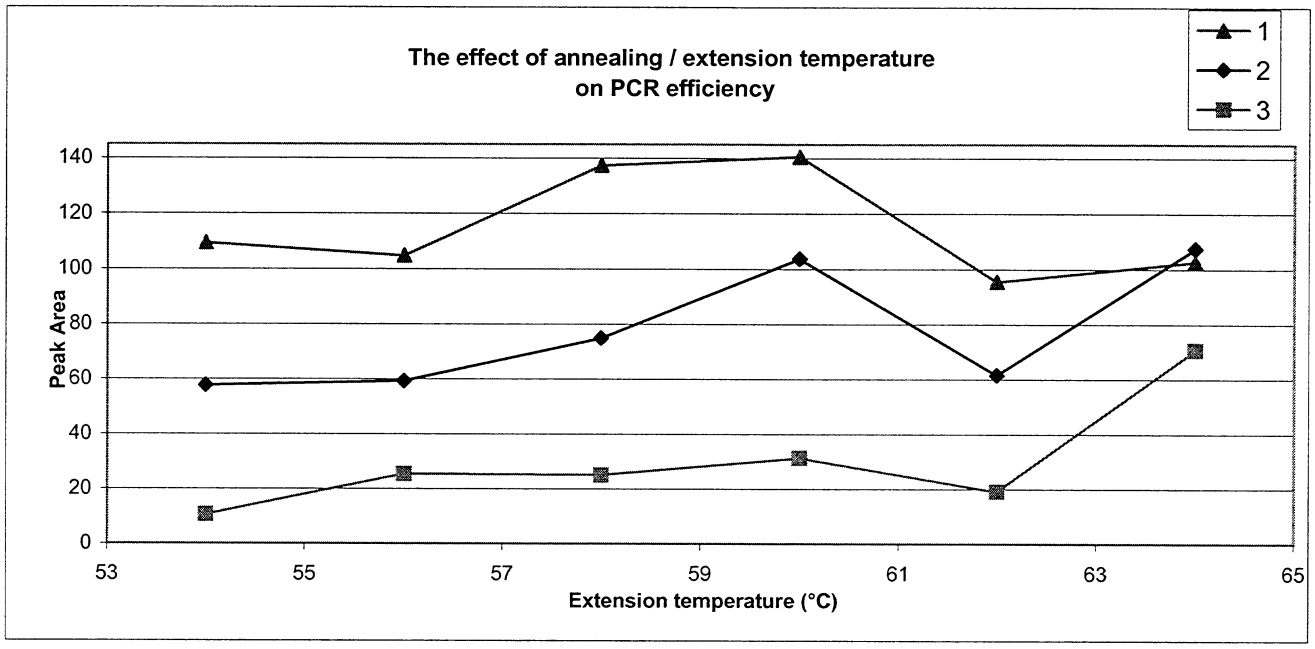

Figure 4: Results of the annealing/extension temperature study. Legend: 1: capillary positive control, 2: capillary covered with Blue Tack^{Tm}, 3: uncovered capillary resting on top of the sample holding block. The reference has an optimum temperature of 60°C. There is a strong efficiency loss in the polymerase chain reaction when the heat transfer is impaired. Results suggest that good contact conditions have a greater influence on PCR efficiency compared to a good thermocycling environment.

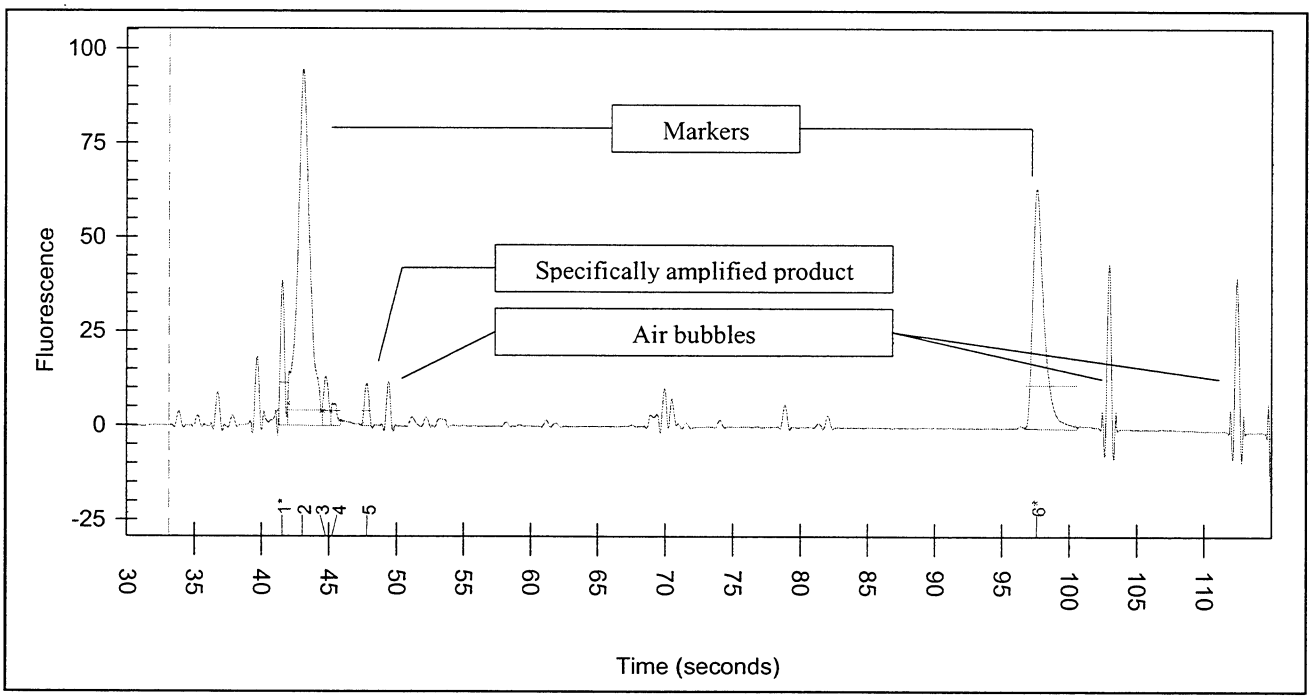

Figure 5: Electropherogram of the proof-of-principle sample analysis. A specifically amplified product is detected (peak 5) after 47.80s. The concentration is determined to be 0.36ng/µl and the DNA strand length is 54bp.