The specificity, sensitivity and efficiency of the PCR microsystem based on LTCC technology

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Abstract: - Polymerase Chain Reaction (PCR) is an enzymatic reaction for specific DNA fragments amplification carrying out by DNA polymerase. PCR is a sensitive and specific tool used in wide range of applications. Various modifications of this technique like incorporation of fluorescence dyes extend the utility of PCR. Classical PCR equipments are extremely sophisticated and thus expensive. Miniaturization of PCR platforms stands for excellent solution to make this technique available for everyone and thus extends the possibility of PCR usage worldwide. The aim of this paper was to compare specificity, sensitivity and efficiency of the PCR performed with classical PCR equipments used routinely in molecular laboratories with the results obtained based on the LTCC PCR microsysytem.

Key-Words: - PCR, Real-Time PCR, Miniaturization, Thermal cycler, Ceramic microchip, Electrophoresis

1 Introduction

Polymerase Chain Reaction (PCR) is an enzymatic reaction for specific DNA fragments amplification carrying out by thermal stable enzyme – DNA polymerase. The process runs periodically and each cycle is composed of three stages as follows (1) double strand DNA denaturation, (2) primers annealing and (3) DNA elongation based on template DNA. The number of cycles might differ and generally in PCR each cycle is repeated 25-40 times. Theoretically after 30 cycles a billion copies of specific DNA fragments might be generated [1].

PCR is a sensitive and specific tool used in wide range of applications, mainly in biotechnology, clinical and forensic medicine, microbiology, biochemistry etc. The interests and utilities of this technique are still growing. Various modifications of this technique extend the utility of PCR.

The classical PCR requires pair of primers, polymerase, mix of nucleotide and template DNA. Incorporation of fluorescence dyes into this technique enable for simpler PCR product detection or even for PCR products quantification.
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miniatrization of PCR platforms stands for excellent solution. Miniaturization is becoming very popular in a variety of scientific disciplines as well as in industry [3], [4], [5].

The LTCC (Low Temperature Cofired Ceramics) technology is becoming very popular recently in various branch of industry, including biomedicine. One of the most important advantages of ceramics is highly resistant to various chemical compounds and substances even to concentrated acids [6]. Furthermore some types of ceramics are non-toxic to PCR components, especially to DNA polymerase [7]. Moreover, ceramics possess others numerous advantages like relatively low price, short time of a new structure development and compatibility microelectronic technique. For these reasons ceramics become widely used in military, data processing or medicine [7], [8]. Considering all mentioned above we have proposed the LTCC-based PCR microreactor to apply in biotechnology.

We have recently described the ceramic microchip in LTCC technology for PCR applications [7, 10, 11]. We have shown that PCR can be successfully performed on the ceramic microchip. In present paper we are applying the ceramic microchip for the various PCR approaches that are widely used in molecular laboratories. Furthermore, in this paper we are comparing specificity and sensitivity of the PCR results performed with classical PCR equipment used routinely in molecular laboratories with the results obtained based on ceramic PCR microsystem. The aim of present paper is also to assess the efficiency of the PCR with the use of LTCC microsystem in comparison to classical PCR devices.

2 Reagents, equipments and PCR conditions

2.1 Commercial PCR equipments

Three commercially used PCR machines were applied for the studies. Two of them were classical PCR devices for end-point PCR product detection, that is: (1) GeneAmp PCR System 9700 (Applied Biosystems) and (2) Eppendorf Mastercycler (Eppendorf). The third tested thermal cycler was dedicated to Real-Time PCR approach with concurrent PCR product detection during the PCR process: ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems).

2.2 PCR and its modification

Three various PCR modifications were applied for the LTCC PCR microsystem and results were compared with commercial PCR equipments mentioned above. The end-point detection systems relied on PCR product detection either by capillary electrophoresis using LIF (Laser Induced Fluorescence) technology or classical agarose-gel electrophoresis. The third PCR approach applied for study was Real-Time PCR based on TaqMan probes.

2.2.1. End-point PCR systems

The end-point PCR systems were performed with use of Qiagen Multiplex PCR Kit (Qiagen) according to manufactured protocol. The single modification of the protocol was to decrease total volume to 10 µl. The pairs of 2 µM primers Forward and Reverse were used. Two approaches were applied: LIF technology and classical PCR with detection by agarose-gel electrophoresis. LIF technology required F primer labeled by fluorescence dye at 5’ end. Different dyes were used resulting with different extinction wave length and thus giving different light color. Numerous miniSTRs (polymorphisms of mini Short Tandem Repeats) markers were amplified: D13S317 (88-132 bp), D3S1358 (72-120 bp), D21S11 (153-211 bp), D16S536 bp), D5S818 (81-117 bp), Amelogenin (57 bp female, 57-63 bp male, (bp – base pairs)). The expected PCR product sizes differed depending on the alleles distribution. The PCR products were further analyzed by capillary electrophoresis. The classical PCR was performed according to protocol described by Qiagen Multiplex PCR Kit, but with total volume 10 µl. 2 µM pairs of primes amplifying fragment of genomic DNA were used without any modification. PCR products were detected using 2% agarose-gel electrophoresis in 1xTBE buffer and visualized by ethidium bromide. The total times of PCR reactions for both approaches with the use of the commercial thermalcycles and microsystem were similar and lasted about 2.5 hours.

2.2.2 Real-Time PCR system

The real-time PCR was performed with the use of various reagents and kits and results were compared. Following reagents were used: (1) TaqMan Fast Real-Time PCR MasterMix (Applied Biosystems), (2) Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies), (3) Precision™ 2x Real-Time PCR Mastermix (PrimerDesign), and (4) FastStart TaqMan Probe Master (Roche). The real-time PCR conditions were adjusted according to manufactured protocols, the total volume applied for the ceramic PCR microchip was 10 µl. The concentration of primers and probe varied and was...
adjusted experimentally. The best results were seen for 10 µM pairs of primers (F and R) and 5 µM probe. The probe was labeled by FAM at 5’ end and BHQ-1 at 3’ end. The total times of reactions for both devices were similar and lasted about 40 min.

2.3 PCR products detection
The PCR products were detected differently depending on the technology. The products of classical end-point PCR were detected by 2% agarose-gel electrophoresis in 1xTBE buffer under 100 V and visualized by the presence of ethidium bromide in UV light and analyzed with the use of KODAK MI (Molecular Imagine Software) v.4.0.0. The PCR products utilizing LIF technology were detected by capillary electrophoresis together with GeneScan® LIZ500 Size Standard using ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Results were analyzed by GeneMapper ID, v.3.2 and presented as electropherograms. The Real-Time PCR products were analyzed during the reaction by SDS software v.2.2.2 and presented as an amplification plot. The Real-Time PCR products performed on the ceramic microchip were analyzed as an end-point PCR by agarose-gel electrophoresis.

3 Results
3.1 Classical end-point PCR
There was no visible difference between results obtained after classical PCR performed on commercial thermal cyclers and PCR performed on ceramic microreactor. The PCR products were specific and the PCR efficiency was similar for each used thermal cycler. Design protocol was to obtain routinely amplified genomic DNA fragment giving PCR product of 384 bp in length. For each device specific product was received with no additional unspecific products (no additional bands were seen). Furthermore, the efficiency of PCR seemed to be similar, the bands of DNA fragments amplified on ceramic microreactor were comparable with bands obtained by commercial thermal cyclers. The examples of PCR products detected by agarose-gel electrophoresis for ceramic microchip and commercial device are presented on fig. 1.

![Fig. 1. Results of the classical PCR performed on ceramic microreactor (A) and on commercial thermal cycles (B). 1 – size standard, 2-4 and 2-6 PCR products, respectively.](image)

3.2 End-point PCR results using LIF technology
We have obtained similar results for the PCR using LIF technology for ceramic microchip when compared with commercial thermal cyclers. Numerous routinely amplified fragments with primer F labeled by fluorescence dyes were amplified by commercial thermal cyclers and ceramic microchip. The electropherograms of PCR products were similar. For this application the PCR product was also specific; no additional picks standing for unspecific PCR product were detected. The AUC (Area Under Curve (pick)) reflects to the PCR efficiency. There was no significant difference between AUC value of PCR product obtained by ceramic microchip and commercial thermal cyclers. For present study following DNA markers were amplified: D13S317 (88-132 bp), D3S1358 (72-120 bp), D21S11 (153-211 bp), D16S536 (81-121 bp), D5S818 (81-117 bp), Amelogenin (57 bp female, 57-63 bp male). As an example (see fig. 2), two amplified DNA markers are presented, one labeled by FAM fluorescence dye corresponding to D5S818, the second one by VIC corresponding to D3S1358, giving blue and green pick, respectively.
Fig. 2. Electropherograms of the PCR products using LIF technology performed on ceramic microreactor (A) and on commercial thermalcycles (B). I – Fluorescence dye: FAM - D5S818 (81-117 bp), II - Fluorescence dye: VIC - D3S1358 (72-120 bp).

3.3 Real-Time PCR
Created ceramic microchip was tested as well in terms of Real-Time PCR application in fast format. The fast system enables amplification of specific DNA fragments in less than 40 min. The cycles are very short (about 30 seconds each). For that reason, the equipment needs to cool down and heat up the reaction mixture relatively quick. Furthermore it is crucial to keep set temperature constant with minimal changes.

At the moment we were tested the microchip in terms of ability to give positive results using fast Real-Time PCR approach. In future In future ceramic microreactor will be equipped with an optical system reading the fold of changes in fluorescence intensity during reaction. At the moment we were tested the microchip in terms of ability to give positive results using fast Real-Time PCR approach.

We run reaction on commercially used Real-Time PCR platform and on ceramic microchip. The reaction was composed of 40 cycles, 25 s for each cycle. We have obtained specific results from both types of devices the Real-Time PCR platform and the ceramic microchip. The PCR products from the microchip were detected by agarose-gel electrophoresis and the products were specific possessing expected size that is 247 bp.

Adjusting the optical system for the ceramic microreactor in the future will enable to performed Real-Time PCR in concurrent PCR product detection and quantification. In present paper we have proved that the ceramic microchip can be applied for the fast Real-Time PCR successfully. The example results of the Real-Time PCR approach are presented on fig. 3.
4 Conclusion

Miniaturization is becoming very popular in a variety of scientific disciplines as well as in industry. Many advantages of miniaturization like low price of analysis, mobility, easy with handling and using make this technology more popular. The classical PCR equipment are is sophisticated and expensive. For that reason the analysis based on PCR are performing by specific laboratories. In order to meet the demand of PCR use, the miniaturization of PCR platforms stands for excellent solution.

Presented PCR microreactor manufactured in LTCC technology stands for the miniaturized PCR platform for the various PCR modification. Three main PCR techniques were tested using the ceramic microreactor. For each PCR application obtained results were similar to the results received by commercial, routinely used thermal cyclers with no significant differences. We have shown that obtained amplified products were specific (we did not notice any unspecific PCR products). We have also observed similar efficiency of PCR performed on commercial thermal cyclers and PCR performed on microchip. Furthermore, we were able to amplify the DNA fragments in a fast PCR system, in less than 40 min.

Concluding, created by us ceramic microreactor can be widely used for various PCR applications with specificity and efficiency comparable to commercially used PCR platforms.

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