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On-chip PCR amplification of very long templates using immobilized primers on glassy surfaces

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Abstract

In this paper we describe a novel method for visualizing very long DNA fragments (for example >6 kb) which are difficult to spot with commonly used arrayers or capillary samplers with very small nanoliter volumes, using directly bound primers on "on-chip" polymerase chain reaction (PCR).

We have used the genomes of the M13 bacteriophage (7.2 kb) the human mitochondrion (16.5 kb) as examples of long DNA templates to test the PCR and were able to elicit robust reactivity. Over 75% of the immobilized primers could be elongated to their fullest extent.

In addition we were able to elicit the PCR reaction with double stranded templates in which one primer was immobilized and the other suspended in the reaction solution.

These synthesized PCR products were visualized by either confocal microarray scanning or fluorescence microscopy using Cy5-dye fluorescence of the modified free primer, or the fluorescence of intercalating dyes.

Keywords: PCR amplification; DNA templates; Immobilized primers

1. Introduction

Advances in "integrated analysis systems" technology are now directed towards point of care diagnostics and the development of the required lab-on-chip devices, which are capable of minimizing the volume requirements for biochemical and chemical reactions. The lab-chip currently available is the micro-total-analysis system (μTAS), which uses miniatured channels to integrate different reaction chambers with methods of analysis (Kakuta et al., 2001). Woolley et al. (1996) described PCR within a chip and there is an increasing trend in biosensors to combine biochemicals with localized reaction partners. As a result, on-chip biochemical reactions and analysis is an accepted approach for lab-on-chip technology. It has been suggested that the performance of lab-on-chip analytical process could be significantly enhanced by localizing more complex biochemical reactions on defined sites on a chip. This idea has recently been tested using restriction enzymes (Bier et al., 2002a, 2004), where the enzymes simultaneous act on a variety of immobilized oligonucleotides. It also has been shown that DNA-polymerases are able to process surface bound templates and primers (Buckle et al., 1996), for example, detection of telomerase activity in PCR-free cell extracts (Schmidt et al., 2002).

Despite these advances in lab-on-chip technology in the past decade, including the localization of PCR by use of either gel pads (Strizhkov et al., 2000) or gel layers (Mitra and Church, 1999), and the demonstration by Erdogan et al. that single stranded templates are necessary for PCR elongations on immobilized primers, extending the length of the templates above 5.7 kb (Erdogan et al., 2001), still remains a technically demanding exercise.

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A number of applications require very long DNA fragments (i.e. >6 kb) to be coupled locally to a surface. One such application is our nanotechnological method of pattern formation by nucleic acids (Bier et al., 2002b; Hölzel et al., 2003). It is technically challenging to spot larger DNA fragments (>6 kb) with common arrayers or capillary stamplers, using very small volumes in the range of nanolitres or less. This is because, due to the high compression, the molecules tend to coalesce in these small volumes, resulting in the clogging of the capillary. To overcome these technical obstacles, we have developed a on-chip PCR for very long templates with directly bound primers. With this PCR technique we have been able to synthesize complete plasmid molecules and complete human mitochondrial genome molecules from genomic DNA up to 16.5 kb.

Two coupling methods have been investigated, first, the affinity bridge via biotin/avidin (specifically neutravidin) and, second, the covalent coupling using *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimid (EDC)-bound primers.

In this paper we have shown that the whole polymerase chain reaction (PCR) process can be performed with one directly and exclusively immobilized primer, which is presented to the reaction partners in a liquid phase.

2. Materials and methods

2.1. Template preparation

Human genomic DNA was extracted from white blood cells (buffy coat) using whole blood samples. Commercial plasmid-DNA (Fermentas, Vilnius, Lithuania) of M13 bacteriophage was propagated in *E. coli* and prepared with standard plasmid preparation protocols.

2.2. Primer construction

The mitochondrial primers were constructed by an alignment (Higgins and Sharp, 1988) of human, chimpanzee and gorilla sequences (Genbank Acc.-Nos.: J01415, X93335, X93347) out of the large ribosomal RNA gene (16S). Two primers of a highly conserved region were selected. The primer sequences are:

Forward primer:

5′-GAGTTCAGACCGGAGTAATCCAGGTCGG-3′ (f-A)

Reverse primer:

5′-$(GA)_{10}$-GCTGCACCATCGGGATGTCCTGATCC-3′ (r-A)

The plasmid primers of the M13 bacteriophage were selected with regard to other experiments (see Hölzel et al., 2003). The sequences of the primer are:

Forward primer:

5′-Cy5-TTTCGCGCTTGGTATAATCGCTGG-3′ (f-B)

Reverse primer:

5′-$(GA)_{10}$-ACCAGCGCTAAAGACAAAAGGGCGAC-3′ (r-B)

Alternative forward primer for a 652 bp fragment:

5′-Cy5-GGGGGCATTAACTGTTTATACGGGC-3′ (f-C)

The forward primer had a Cy5-modification (Molecular Probes, Portland) at its 5′-end for detection of the complete synthesis product.

For immobilization the reverse primers (r-A, r-B) were either biotinylated or phosphorylated at the 5′-end after synthesis (Carl Roth GmbH, Karlsruhe).

2.3. Covering of the chip surface with neutravidin

The surface of the glass chips (cover slides) was activated with 5 N NaOH for 2 h and rinsed in ddH_2O for 30 s and directly incubated in a 10% 3-aminopropyl-triethoxysilane (APTES, Fluka, Seelze)-water solution (pH 3.45/HCl) for 2 h at 80 °C. Finally the chips were rinsed again in ddH_2O for 30 s and dried at 120 °C for 1 h. In a moist chamber 100 μl of neutravidin (0.2 mg/ml) in water were dropped on the slide, which was incubated for 2 h at room temperature (RT). The neutravidin (Pierce, Rockford) solution was rinsed for 30 s in ddH_2O and the water residues were spun off for 10 min at 400 × *g*. The ready for use chips were stored dry.

2.4. Covalent coupling using methylimidazol/EDC (Violeta and Swaisgood, 1982)

A 50 μM stock of 5′-phosphorylated primers was diluted twice with a 10 μM methylimidazol solution (Fluka, Seelze) (pH 6) followed by an 1:4 (w/v) addition of *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimid (EDC, Applichem, Darmstadt) and 5 min incubated under smooth shaking at RT. After incubation the solution was directly spotted on APTES-silanized chip-surfaces using a contact-free arrayer (Nanoplotter, GeSiM, Dresden). The freshly spotted array has to be incubated in a moist chamber for at least 4 h. At last the array was blocked by overlaying the chip surface with blocking reagent (2 g succinic anhydride in 50 ml dimethylformamide (DMF, Fluka, Seelze)) for 30 min at 37 °C and subsequently washed in Tris/HCl (pH 8) (Applichem, Darmstadt) and water each for 10 min and dried at RT.

2.5. Spotting of primer

The activated primer, either biotinylated or phosphorylated, were deposited on the prepared surface by use of a piezo-pipette armed robot, the “Nanoplotter” (GeSim, Dres-