SINGLE MOLECULE AMPLIFICATION IN A CONTINUOUS FLOW LABCHIP DEVICE

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Abstract

We have developed an integrated system to perform automated, high-throughput, continuous flow PCR in a microfluidic sipper chip. The system is unique in several respects. One, the system integrates reaction assembly, thermal cycling and fluorescence detection on one chip. This allows different samples to be tested one after another in an automated way, all at nanoliter scale. Two, the chip we have created uses our sip-and-split design and has eight channels in which eight different loci can be amplified at one time for each sample. In addition, we have recently configured the system to continuously amplify and detect single molecules of DNA

Keywords: cancer diagnostics, genotyping, PCR, single molecule analysis

1. Introduction

New biological and diagnostic markers are rapidly emerging from genomic studies that can provide meaningful insights into a patient's health. In many cases, these involve the analysis of nucleic acids. For complex genomes, the polymerase chain reaction (PCR) is often used to prepare the sample for sequence-specific or allele-specific interrogation [1]. The advantages of "lab-on-a-chip" devices, i.e. miniaturization, integration and automation, would be useful additions to the diagnostician's arsenal as they produce better data quality, reduced cost, and improved ease-of-use features by comparison to conventional technology. An emerging need in nucleic acids diagnostics is detecting rare mutant molecules from conveniently obtained patient specimens that reflect the presence of neoplastic tissue [2]. Recently, Vogelstein and Kinzler described an approach called Digital PCR whereby individual molecules are genotyped, allowing rare molecules to be detected in the presence of a high concentration of molecules bearing the wild type sequence [3]. Here we present an automated, microfluidic system capable of analyzing single molecules by PCR amplification and TaqMan genotyping. The integrated features of this system make it a candidate format for high throughput, diagnostic laboratory settings.

2. Experimental

The chip shown in Figure 1 is made of two pieces of quartz. One piece of quartz has channels etched into it, and the other has nine metal traces deposited on it and through-holes that serve as fluid reservoirs. These plates are then bonded together, which creates

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fluid conduits terminating at the reservoirs that can be loaded with reagents. A capillary, or "sipper", is inserted into a hole such that its lumen intersects with the channel network and is then used to access samples automatically from a microtiter plate. The volume of DNA sipped is typically 1-2nL and the total reaction mixture is approximately 10nL. DNA samples are brought up through the sipper, mixed with Taq Polymerase and dNTPs, and split into 8 parallel channels. Each channel is supplied with a set of locus specific reagents (primers, probes and $MgCl_2$), which are added immediately prior to the heated zone on the chip. Between each sample, the sipper accesses buffer from a trough on the instrument. The buffer trough serves to both wash the sipper and to introduce a buffer spacer to separate one DNA sample from the next. The reaction mixture and buffer spacers are pulled by vacuum through the portion of the chip that contains the metal traces that are powered with electric current to produce heat. As the reaction mixture moves through the channel, it is heated and cooled, at a very rapid rate, to amplify the DNA. Typical cycle times are 5 seconds for denaturation, 7 seconds for annealing, and 5 seconds for extension. The flow rate is set to allow each reaction mixture to see the desired number of amplification cycles. The fluorescence signal generated by the amplification and exonucleolytic activity is detected near the end of the fluidic channel. All eight fluid paths are identical in length and hydrodynamic resistance.

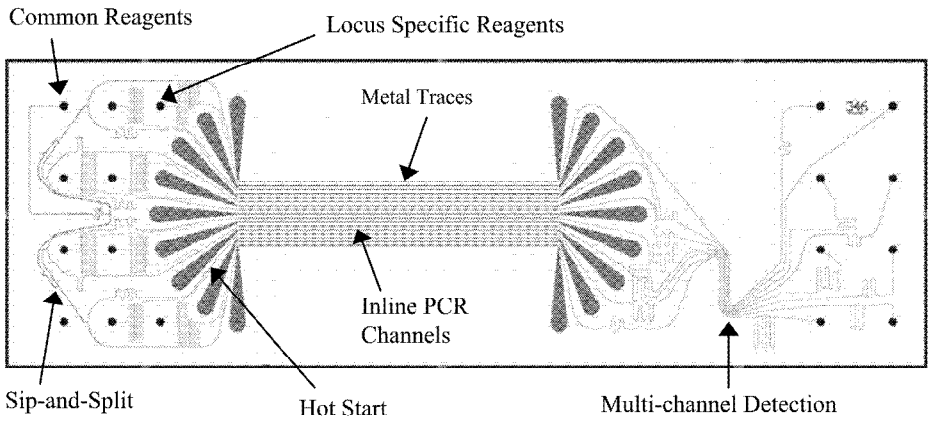


Figure 1. Chip design with 8-channels for reaction assembly, PCR and detection

3. Results and discussion

This integrated system produces robust amplification of small volumes of genomic DNA. As shown in Figure 2, the fluorescence signal generated from thousands of PCR reactions is extremely reproducible. In this 18-hour run, all no-template-controls are negative; there are no failed PCR reactions and no contamination.

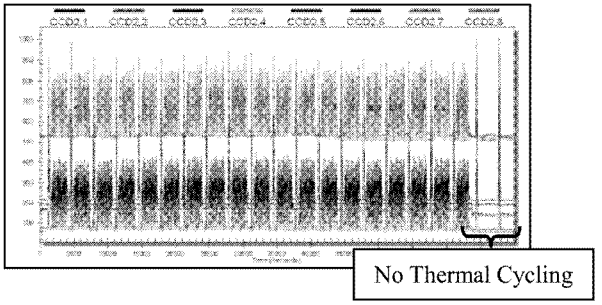
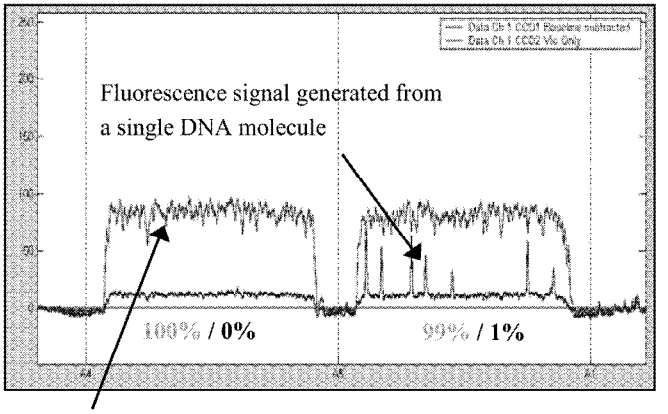


Figure 2. Data trace demonstrating amplification and detection of genomic DNA for 18 hours

Figure 3 shows how this system can accurately amplify and detect rare molecules within a large population. As a model, we mixed two DNA samples, each homozygous for the two alleles of a SNP. In this experiment, we were able to detect single molecules for one allele that were present in a large population of molecules of the other allele (7 in 700 for this case). The characteristic peak shape from molecule to molecule is evidence that both PCR and dispersion of the resulting fluorophores are very reproducible.



Fluorescence signal generated from (1) 100% or (2) 99% of the amplified molecules (~700 total)

Figure 3. Data trace from a single molecule amplification reaction on-chip.

To demonstrate the relevance of this microfluidic PCR system to cancer diagnostics, we have used it to analyze cancer patient samples and detect rare mutant molecules of k-ras, a gene diagnostic for colon cancer (Figure 4). The data trace shows fluorescence at

two wavelengths vs. time for one of the eight fluid channels. Two TaqMan probes, one specific for the normal allele, and one specific for the mutant allele, is the detection method. All molecules but one in the data trace show a large gray peak and a small black peak. These peaks are produced by the allele-specific (gray) and background (black) TaqMan probe cleavage surrounding the amplification products of normal genomic DNA molecules. When a mutant molecule traverses the system, it is amplified and recognizable as the large black peak. There are 15 individual molecules in this data trace spanning 3000 seconds, all contained within 200nL of fluid.

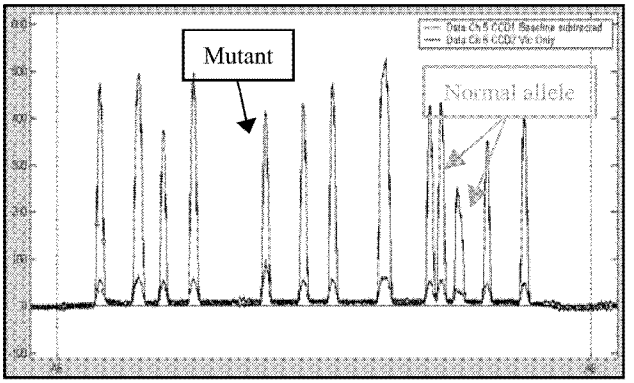


Figure 4. Detection of mutant molecules purified from patient stool samples

4. Conclusion

The features of automated sample accession, microfluidic reaction assembly, integrated thermal cycling, and associated optical spectroscopy have been used to create a nanoliter scale nucleic acids analyzer. By controlling the input DNA concentration, it is possible to get genetic information from single, mutant molecules present among large numbers of wild type molecules. This capability enables the analysis of conveniently obtained patient specimens for the presence of important diagnostic markers.

References

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