Miniaturized Genetic-Analysis System

Rolfe C. Anderson, Gregory J. Bogdan, and Robert J. Lipshutz
Affymetrix, Inc., 3380 Central Expressway, Santa Clara, CA 95051

ABSTRACT

We describe a miniature cartridge for carrying out all required DNA target-preparation reactions for hybridization-based sequence analysis in 10 μ L volumes. Using this cartridge we demonstrate a hybridization-based HIV-1 mutation assay, including the polymerase chain reaction (PCR) [1], in-vitro transcription (IVT), labeling, fragmentation, hybridization, scanning and data analysis with performance equivalent to benchtop assays.

INTRODUCTION

Chemical and biological assays employing a series of operations such as mixing, reacting, separating, and sensing are critical in numerous fields including clinical diagnostics, biomedical research, analytical chemistry, industrial process control and environmental monitoring. Existing methods are based on macroscopic, bench scale reactions and operator intervention and are not amenable to reducing sample and reagent volumes, labor, processing time, and cost or improving reliability and portability. Miniaturizing, integrating and automating these assays will achieve these goals and result in broader applicability. Many investigators have reported miniaturizing analytical systems [2]. Single reactions such as DNA amplification by PCR in microfabricated chambers [3] and analysis steps such as capillary electrophoresis on glass substrates have been demonstrated [4] [5]. Systems in which mixing is followed by separation have also been demonstrated [6].

The elucidation of the DNA sequence of all human genes is creating new opportunities in genetic analysis with applications in clinical diagnostics, biomedical research, and pharmaceutical discovery. Genetic analysis typically requires nucleic acid extraction, amplification, labeling, and sensing. These complex assays are clear candidates for miniaturization and

integration.

Hybridization-based genetic analysis using high-density DNA probe arrays has been demonstrated [7] [8] [9] [10]. These microfabricated DNA arrays may contain more than one million different DNA probe regions and have sufficient complexity to analyze tens of thousands of nucleic acid bases. These arrays have been used to detect mutations associated with cystic fibrosis and hereditary cancers in human samples, and mutations in HIV putatively associated with drug resistance. Data from a DNA probe-array assay is shown in Figure 1.

The hybridization-based HIV-1 mutation assay is described in Figure 2. Currently, these manual steps are individually carried out at a laboratory bench in 20 to 1000 μ L volumes. Any program to miniaturize and automate this assay must address biocompatibility between reagents and the chamber walls, thermal isolation of sensitive reagents from the high temperature reaction chambers, and the quantitative transfer of fluids between chambers. This preliminary report describes a miniature cartridge to automatically perform these steps in 10 μ L volumes.

REACTION MINIATURIZATION

We are following a natural progression from reactors to systems, first individually demonstrating each reaction in miniaturized chambers, and then combining these reactions into a system. This progression allows us to address reactor biocompatibility before fluid movement, fluid control and thermal isolation. Reactors connected with channels were fabricated from polycarbonate.

Figure 1. Fluorescence image of microfabricated 1.28 x 1.28 cm HIV-1 DNA-array chip after hybridization with test RNA sample. The array contains over 20,000 88 by 91 micron features.

Figure 2. Process steps in the hybridization-based HIV-1 mutation assay. The temperature-cycled PCR reaction selectively amplifies the DNA region of the HIV-1 genome to be interrogated. T3 or T7 RNA Polymerase transcribes the DNA template yielding fluorescently labeled RNA. Thermal fragmentation randomly breaks the RNA into lengths appropriate for hybridization. The fragmented RNA is incubated with the DNA probe array so that fragments may bind (hybridize) with complementary sequences on the array. After hybridization, the fragments that are not hybridized are washed away. The array is read by epifluorescence confocal scanning.

Figure 3. Top view of an array of polycarbonate reaction chambers. Dimensions are channel: 250 μm wide by 125 μm deep; reactors: 1.5 mm wide by 13 mm long by 125 to 500 μm deep, with reactor volumes from 2.5 to 10 μl .

Polycarbonate can be injection molded, ultrasonically welded, sterilized, manufactured at low-cost, and provides low thermal conductivity.

A top view of an array of polycarbonate reactors is shown in Figure 3. As shown in the cross section, Figure 4, the lower wall of the chamber is a thin sheet of polycarbonate. The reactor temperature is controlled by contacting a thermoelectric device with this thin wall (Figure 5).

PCR in a reaction chamber was performed using a standard biochemical and thermal cycling protocol [11]. A dedicated computer monitored and controlled the temperature as it was cycled between 55°C, 65°C and 94°C (Figure 6). Evaporation and bubble collapse were addressed by ensuring a clean reactor and bounding the reaction mixture with a hydrophobic

Figure 4. Cross section of a polycarbonate reactor.

Figure 5. System for controlling reaction temperature with measurement using a resistive temperature device (RTD).

Figure 6. Labview-based controller for PCR reaction with target and actual temperature-time data for microreactor.

fluid. Thirty five thermal cycles resulted in billion-fold amplification in a 13 μl sample, appropriate for subsequent reaction steps (Figure 7). The single narrow band on the gel and subsequent hybridization tests demonstrate excellent amplification fidelity.

Transcription and labeling were performed using a standard in-vitro transcription reaction to copy the DNA to RNA while incorporating bases with attached fluorescein labels [12]. Sensitivity to contamination by environmental RNA-destroying enzymes (RNase), and denaturation of the transcription enzyme (RNA polymerase) were addressed by beginning with and maintaining sterile conditions, and slightly increasing the enzyme concentration. The reaction was carried out in the polymeric chamber in a 3 μl volume with a 70% yield, compared to a control reaction (Figure 8).

The fragmentation reaction requires heating the labeled RNA to 94°C in the presence of Mg^{++} [13]. Temperature was controlled using the configuration shown for PCR. Evaporation was controlled by sealing the ends of the reaction chamber with a hydrophobic fluid. Purified labeled RNA was used to monitor fragmentation with agarose gel electrophoresis. Miniature and benchtop fragmentation gave equivalent

Figure 7. Agarose gel results for 35 cycle PCR amplification of 1100 base portion of HIV-1 from 2000 starting copies. Reaction in microchamber yielded 10^9 amplification, appropriate for subsequent reaction steps.

hybridization results.

SYSTEM INTEGRATION

Effectively combining the reactions described in Figure 2 into a single system requires fluid position control, liquid dosing, mixing, and thermal isolation between adjacent chambers. The RNA-polymerase enzyme would be denatured at the high temperature (94°C) used in the PCR and fragmentation reactions. Thermal isolation is accomplished by fabricating the reaction and storage chambers more than 10 mm apart in a thin polycarbonate reactor and by controlling the temperature in each region using thermoelectric devices.

In this assay the PCR product must be measured and mixed with an IVT cocktail stored in the same cartridge (Figure 9). The cartridge design and fluid-movement algorithm for this series of reactions are shown in Figures 3 and 10, respectively. Performing this series of reactions in the cartridge resulted in a 30% yield for the PCR reaction, and a 15% net yield through IVT, as compared to the control (Figure 11). This product concentration is appropriate for the subsequent hybridization reaction. In this case, less dilution of the fragmentation mixture prior to hybridization is required versus the standard benchtop protocol.

Figure 8. Agarose gel results indicating that the transcription reaction carried out in microchambers with a 3 μ l volume has 72% yield as compared to the control reaction performed in a conventional chamber.

Figure 9. This HIV-1 target preparation protocol requires measurement and mixing of two liquid streams.

Figure 10. Series of fluid movements used to measure part of the PCR product and combine it with the IVT mixture. Oscillation at step 4 achieves mixing.

Figure 11. Agarose gel results for HIV-1 assay reaction sequence (Figure 2) demonstrated on a single device.

CONCLUSIONS

We have developed a genetic analysis system that integrates all of the preparative reactions necessary for a hybridization-based HIV-1 mutation assay with overall performance comparable to the bench-top protocol. Further optimization of this system will provide greater yields and improve sensitivity. This work comprises a crucial step towards a fully integrated genetic analysis system. We are following an evolutionary path, first developing disposable polymeric cassette-based reaction systems, and later, where appropriate, incorporating microfabricated elements. This approach has allowed us to quickly generate a working system and address materials compatibility issues in reaction volumes equal to or smaller than those published for silicon and glass devices.

The specific protocol we have demonstrated can be extended to other assays that employ hybridization-based sequence analysis by using different temperatures, other fluidic movement schemes, and other reagent combinations. Moreover, the components we have developed and the approach we describe can be applied to other genetic-analysis protocols and biochemical assays in general.

ACKNOWLEDGMENTS

The advice and assistance of scientists at Affymetrix were invaluable in performing these experiments. The authors would like to particularly thank Nila Shah, Maureen Cronin, Tom Gingeras, and Huu Trann for their patience, helpful advice, and for providing certain reagents. Richard Rava provided valuable scientific guidance and comments on this manuscript. This work was supported by NIST cooperative agreement 70NANB5H031.

REFERENCES

1. R.K. Saiki, et al., "Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia", *Science*, 230: 1350-1354 (1985).
2. *Proceedings of the μ TAS '94 Workshop*, A. van den Berg and P. Bergveld, ed., Kluwer (1995).
3. M.A. Northrup, et al., *Technical Digest of Transducers 1993, Seventh International Conference on Solid State Sensors and Actuators*, Yokohama, Japan, (1993).
4. D.J. Harrison, K. Fluri, K. Seiler, Z. Fan, C.S. Effenhauser and A. Manz, "Micromachining a Miniaturized Capillary Electrophoresis-Based Chemical Analysis System on a Chip", *Science*, 261, 895-897 (1993).
5. A.T. Woolley and R.A. Mathies, "Ultra-high-speed DNA fragment separations using microfabricated capillary array electrophoresis chips", *Proc. Natl. Acad. Sci.*, 91, pp. 11348-11352 (1994).
6. D.J. Harrison, K. Fluri, Z. Fan, and K. Seiler, "Integration of Analytical Systems and Incorporating Chemical Reactions and Electrophoretic Separation", *Proceedings of the μ TAS '94 Workshop*, p.105 (1995).
7. S.P.A. Fodor, J.L. Read, M.C. Pirrung, L. Stryer, et al. "Light Directed, Spatially Addressable Parallel Chemical Synthesis", *Science*, 251, 4995, pp. 767-773 (1991).
8. S.P.A. Fodor, R.P. Rava, X.H.C. Huang, A.C. Pease, et al., "Multiplexed Biochemical Assays with Biological Chips", *Nature*, 364, 6347, pp. 555-556, (1993).
9. A.C. Pease, D. Solas, E.J. Sullivan, M.T. Cronin, et al., "Light-Generated Oligonucleotide Arrays for Rapid DNA Sequence Analysis", *Proceedings of the National Academy of Sciences*, 91, 11, pp. 5022-5026 (1994).
10. R.J. Lipshutz, D. Morris, M. Chee, E. Hubbell, et al., "Using Oligonucleotide Probe Arrays to Access Genetic Diversity", *Biotechniques*, 19, 3, pp. 442-447 (1995).
11. Reaction mixture consists of 2000 molecules of template DNA, 0.3 units of TAQ polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.2 μ M primers, and 1X Perkin Elmer PCR buffer. Thermal program includes: (1) an initial denature at 94°C for 60 seconds, (2) a denature at 94°C for 20 seconds, (3) an anneal at 65°C for 40 seconds, (4) an extend at 72°C for 50 seconds, (5) steps 2 through 4 repeated 35 times total, (6) a final extend at 72°C for 60 seconds.
12. Reaction mixture consists of 2.5 mM each of ATP, CTP, GTP, and 0.5 mM UTP, 0.25 mM Fluorescein-UTP, 8 mM $MgCl_2$, 50 mM HEPES, 1X Promega Transcription Buffer, 10 mM DTT, 1 unit T3 RNA polymerase, 0.5 units RNAGuard (Pharmacia). Thermal program consists of 37°C for 60 minutes.
13. Thermal program is 94°C for 60 minutes.