A Novel Control System for Polymerase Chain Reaction Using a RIKEN GS384 Thermalcycler

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(Received 21 November 1997)

Abstract

We have developed a novel high-throughput thermalcycler, the RIKEN GS384, which has a maximum of 1536 wells and whose temperature can be controlled accurately and simultaneously for a very small volume of a reaction mixture. In practice, the reaction is carried out using four 384-well (3.5 mm in diameter) plate formats which can be automatically moved using a robotic arm. To achieve accurate temperature control with high thermo-conductivity, we adopted Teflon-coated aluminum well plates closely sandwiched between silicon sheet-covered lids on top and a graphite sheet below. The lids were kept at a higher temperature (2 to 5 °C) than the reaction wells. The temperature of the 1536 sample wells was controlled accurately without temperature variability among the wells or evaporation, even for samples of very small volume (minimum 2 µl). We also developed a new type of plate format which is similar to the 384-well plate in terms of plate size, shape, and material, but which differs in the number (1536) and size (1.6 mm in diameter) of the wells. Since the amplification reactions could be done precisely as well, a total of 6144 reactions can potentially be carried out simultaneously using the GS384 thermalcycler. This is very promising for DNA microfabrication technology. This thermalcycler offers the advantage of high-throughput DNA analysis which should be useful for DNA diagnoses or for the human genome project.

Key words: PCR; thermalcycler; high-throughput; temperature control

1. Introduction

Enzymatic reactions require accurate temperature control for optimal reaction conditions and yield.1 Recently, such temperature control has been achieved using computerized heating incubator systems, especially for small-volume samples requiring many reactions such as the polymerase chain reaction (PCR).2 However, with the rapid development of DNA technology, it is increasingly important to be able to analyze many samples of small volume under accurately controlled temperature conditions.3 In response to this need, we developed a PCR thermalcycler whose temperature can be controlled accurately and simultaneously in all wells, and which can handle many reaction wells for small-volume samples.

2. Materials and Methods

2.1. Instrument design and operation

For the PCR incubator plate, we adopted an aluminum plate (0.7 cm x 8.0 cm x 11.0 cm) with 384 wells which are 3.5 mm in diameter and 5.0 mm deep, with a distance of 4.5 mm between the centers of two adjacent (Fig. 1A). Each well can be used for a maximum of 50 µl of reaction mixture and has U-shaped bottom which is suitable for complete pipetting. The plates can be used repeatedly because the surface of each well is coated with Teflon to prevent non-specific adsorption which can seriously affect the results of PCR. The GS384 thermalcycler is 30.5 cm high, 50 cm wide and 50 cm deep (Fig. 1B) and has four plates (total of 1536 wells) with lids, a heat plate and a peltier element (Figs. 1C and 2). As shown in Fig. 2, graphite sheets 0.15 mm thick are set between the aluminum well plates and the heating plate. Silicon rubber sheets 0.2 mm thick are attached under the lids, which can be opened and closed automatically by computer. Further, to examine the possibility of increasing the number of samples, a 1536-well (1.6 mm in diameter,
Figure 1. GS-384 thermalcycler and the automated PCR system. (A) The 384-well PCR plate (0.7 cm x 8 cm x 11 cm Teflon-coated aluminum plate) was designed to match to the standard 16 x 24 titer plate size. The wells have a volume capacity of 50 μl and a U-shaped bottom suitable for pipetting. The plate has four handles to make it movable by robotic arm. The well surface was coated with Teflon to minimize non-specific adsorption of DNA. (B) Four plates can be simultaneously subjected to PCR reaction in one instrument (1536 reactions). (C) The heated lid and the PCR plate are grasped by the robotic arm.

Figure 2. Schematic representation of reaction module. This machine consists of (1) a heated lid, (2) a silicon rubber sheet, (3) a 384-well PCR plate, (4) a graphite sheet, (5) a heater and (6) a peltier element (cooler). The graphite sheet (0.15 mm) is located between the PCR plate and the heater to maintain high, uniform thermal conductivity. The silicon rubber sheet (0.2 mm) as a gasket is on the backside of the heated lid. The opening and closing of the heated lid is automatically controlled by personal computer.

13 μl in volume) plate was used (Fig. 5A).

2.2. Temperature measurement
The temperature of the well samples was monitored using a Temperature Monitor RL20 (Yokogawa, Japan) with the records being calibrated with a standardization reference of the National Institute for Standards and Technology (U.S.A.).

2.3. PCR amplification of cDNA fragment
PCR was carried out in a well containing 10 μl of the following reaction mixture: 2 x 10 to 2 x 10⁶ copies of mouse reeler cDNA fragment (1278 bp), 0.1 μM of forward primer (5'-ACGTTGTAAAGACGGCCAGT-3') and reverse primer (5'-TAACATTTCCAGGAAACA-3'), 200 μM of each dNTP, 2 mM MgCl₂, 0.5 units of Taq polymerase and 0.06 units of Pfu polymerase as described previously. The PCR conditions for amplification were 96 °C for 2 min, 55 °C for 1 min for the first cycle and 96 °C for 1 min for the following 30 cycles. A portion of the reaction (5 μl) was analyzed electrophoretically using 1% agarose gel containing SYBR Green (0.5 μg/μl).

2.4. PCR amplification of cDNA library inserts
The cDNA library derived from mouse blastocysts was constructed using the CapFinder™ cDNA library construction kit (CLONTECH). A stick of each white colony was transferred into LB-ampicillin medium of the 384-well plate by Q-bot (Genetix) to prepare master plates of the cDNA library. Using a 384-metal pin array, the library of the replica plates copied from the master plates was then transferred to the GS384 aluminum plates for
PCR amplification. The PCR conditions were 96 °C for 2 min, 55 °C for 30 sec, and 72 °C for 1 min for the first cycle, followed by 96 °C for 30 sec, 55 °C for 30 sec and 72 °C for 5 min for 30 cycles.

3. Results and Discussion

3.1. High-throughput design for accurate temperature control

We developed the RIKEN GS384 as a high-throughput apparatus for many PCR reactions with very small sample volumes. Recently, PCR machines with a 384 wells have become available commercially: MJ Research with a 384-well plastic plate and Perkin-Elmer with a 384-well aluminum plate. Here, we adopted four 384-well aluminum plates for high-throughput reactions and high thermo-conductivity, which allow a maximum of 1536 reactions simultaneously (Fig. 1A and B). The wells of the aluminum plates were coated with Teflon, as no PCR amplification was observed without the coating. Also, the coated plates could be reused. These plates can be transferred to another machine using robotic arms, as shown in Fig. 1C.

The heating rate of the entire machine using these aluminum plates was a maximum of 0.456 °C per second increasing from 55 to 95 °C. This shows the good thermo-conductivity of the apparatus. Plastic plates such as polycarbonate and polypropylene can also be used instead aluminum. They were not used here, because the rate of heating and cooling was not sufficient, and they are expensive and require manual operation. In a high-throughput system, all the wells must be controlled accurately to the same temperature. When our aluminum plate was placed directly on the heater plate and heated for 30 sec to 96 °C, chilled to 55 °C and heated again to 72 °C, the temperature variation among the wells was a maximum of 2.2 °C for 96 °C, 0.7 °C for 55 °C and 0.9 °C for 72 °C (Table 1). To achieve higher thermo-conductivity and thermo-uniformity, we tried using a silicon rubber sheet and a graphite sheet between the aluminum plate and the heater plate. When a silicon rubber sheet was used, the temperature variation at 96 °C was up to 1.2 °C and thermo-conductivity decreased from 0.456 °C/sec to 0.372 °C/sec. On the other hand, we succeeded in keeping high thermo-conductivity (maximum 0.47 °C/sec) while reducing the temperature variation to 0.4 °C when a carbon graphite sheet was used (Fig. 2).

In a high-throughput apparatus, many small-volume samples must be treated. One is serious problem is the loss of samples by evaporation. To prevent this, a film of mineral oil is usually laid over the sample solution. This would be a laborious procedure in the case of many samples. Recent PCR machines are designed to prevent such sample loss by keeping the heated lids at approximately 100 °C to 110 °C which is much higher than the actual reaction temperature. To maintain air-tightness, the reaction tubes must be manually sealed with tape. To eliminate this laborious sealing work, we attached a silicon rubber sheet under the lid (Fig. 2) and examined the temperature profiles under the PCR cycle conditions when the lids were heated constitutively at 105 °C (constitutive lid heating) or coordinately to a little higher (2 to 5 °C) temperature (coordinate lid heating) than that of the well samples. As shown in Fig. 3, the temperature of the well could be controlled more finely by coordinate lid heating rather than constitutive heating. The cooling rate was significantly improved and the reaction time was clearly shortened by the coordinate lid heating.

3.2. PCR performance

The performance of the GS384 thermalcycler was examined with respect to well-to-well temperature variety, amplification sensitivity and its efficiency. When PCR amplification was carried out in a 10 μl reaction volume containing 2 × 10⁶ copies of cDNA, the same amount of PCR product was observed in all 48 wells (Fig. 4A). This suggests that temperature variation among the wells was largely reduced in this machine. Apparently, the smallest amount of cDNA template (1278 bp) required for ampli-

|   | Graphite | Silicon rubber | Direct |
|---|----------|----------------|--------|
| A1 | 95.4     | 54.9           | 70.7   |
| D5 | 95.4     | 54.8           | 70.8   |
| F8 | 95.4     | 54.8           | 70.8   |
| H11 | 95.8    | 54.8           | 70.9   |
| MAX | 95.8     | 54.9           | 70.9   |
| MIN | 95.4     | 54.8           | 70.7   |
| MAX-MIN | 0.4 | 0.1            | 0.2    |

The temperature in the samples was measured and recorded with a temperature monitor RL20 (Yokogawa; Japan). Four probes of this recorder were inserted into four wells filled with 40 μl mineral oil. A1, D5, F8 and H11 indicate the location of the well on the 384-well format. The temperature in each well was measured every time the heater of the GS384 was serially heated at 96 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. MAX, MIN and MAX-MIN indicate maximum temperature, minimum temperature and the difference between them, respectively.

Table 1. Thermal conductivity in various materials between a PCR plate and a heating block.

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Figure 3. Temperature profiles of the heated lid and the 384-well aluminum plate during the PCR cycles. The temperature of the well (light line) in the PCR plate and that of the heated lid (bold line) were measured. (A) Constitutive lid heating, the temperature of the PCR plate was not precisely controlled when the lid was continually heated at 105 °C. (B) Coordinate lid heating, the heated lid was controlled at 2–5 °C higher than the temperature of the PCR plate.

3.3. Reduced volume and more reactions

In a high-throughput system dealing with many valuable samples, sample volume must be reduced. However, sample volumes of less than 20 μl are not recommended with commercially available thermal cyclers, because evaporation is more likely to occur when small volumes are used, resulting in an increase in surface to volume ratio that affects the efficiency of the amplification.\(^3\) To determine the minimum reaction volume for PCR amplification in our machine, we carried out PCR of 30 cycles at various volumes from 1 μl to 50 μl including 50 fg/μl of reeler cDNA and analyzed the PCR product (1 μl) by agarose gel electrophoresis. As shown in Fig. 4C, the amplified PCR product could be observed even in 1 μl of reaction mixture, although the amplification efficiency seemed to be reduced. However, efficient amplification was \(2 \times 10^2\) copies in 30 cycles of PCR (Fig. 4B). The amplification was sensitive and specific. Furthermore, we examined how many clones of mouse blastocyst cDNA library are amplified using long PCR\(^6\) which enables amplification of much longer inserts (\(\sim\) 4 kb). The number of clones amplified was 9892 out of 10,752 (92%). These data suggest that the GS-384 thermal cycler offers excellent temperature control, amplification sensitivity and amplification efficiency.

Figure 4. (A) Well-to-well variation of the amplification in the 384-well plate. The reaction was carried out in the 384-well PCR plate in a volume of 10 μl containing \(2 \times 10^6\) copies (1 pg) of mouse reeler cDNA. The 384 samples were cycled 30 times at 96 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min. Forty-eight samples were analyzed on 1% agarose gel. Lane M is lambda HindIII/EcoRI molecular size marker. (B) Detection of PCR products in different amounts of template. Reactions were performed on samples containing the following dilutions of mouse reeler cDNA: \(2 \times 10^6\) copies (lane 1), \(2 \times 10^5\) copies (lane 2), \(2 \times 10^4\) copies (lane 3), \(2 \times 10^3\) copies (lane 4), \(2 \times 10^2\) copies (lane 5), and \(2 \times 10^1\) copies (lane 6). Ten microliters of each reaction mixture was amplified in the GS384 thermal cycler with 30 cycles of amplification (30 sec at 96 °C, 30 sec at 55 °C, and 1 min at 72 °C). (C) Comparison of amplification yields in different reaction volumes. From 1 to 50 μl samples containing 50 fg/μl reeler cDNA were amplified in the GS384 thermal cycler using 30 cycles of amplification. Lanes 1/1, 1/2, 1/5, 1/10, 1/20 and 1/50 show 1 μl from different reaction volumes (1 to 50 μl) was run on 1% agarose gel containing 0.5 μg/μl SYBR Green.
It is exactly the same as the 384-well plate format in size, shape and material and differs only in the number of wells (1536) and their size (1.6 mm in diameter, 13 μl in volume) (Fig. 5A). When the amplification reaction was carried out in 5 μl reaction volume containing 1 pg of template, successful amplification was observed in all sample wells examined (Fig. 5B). Thus, a total of 6,144 reactions can be carried out simultaneously by the GS384, which would enable us to theoretically do 43,008 reactions per day. This GS384 thermalcycler appears to be very useful for large-scale DNA analyses such as in genome projects and clinical diagnoses.

Acknowledgments: We thank Mari Itoh for the technical assistance. N. S. was supported in part by Core Research for Evolutional Science and Technology (CREST) from Japan Science and Technology Corporation. This study was supported by Special Coordination Funds and a Research Grant for the Genome Exploration Research Project from the Science and Technology Agency of the Japanese Government, and a Grant-in-Aid for Scientific Research on Priority Areas and Human Genome Program from the Ministry of Education and Culture, Japan to Y. H.

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