Multichannel PCR and Serial Transfer Machine as a Future Tool in Evolutionary Biotechnology

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ABSTRACT

As an improved strategy for producing functional macromolecules by in vitro *evolutionary optimization, we propose an automated machine that can process up to 960 samples in parallel. It consists of a 960-well PCR machine with special sealed plastic reaction vessels and appropriate handling devices. We show that the heat-sealing technique does not significantly affect the activity of* Taq *DNA Polymerase or that of the temperature-sensitive* Qβ *RNA polymerase but avoids cross-contamination and evaporation of the samples. Initial experiments demonstrate the suitability of the apparatus to uniformly process the samples and to perform the thermocycling. Serial transfer of reaction products into fresh reaction solution was used to initiate further rounds of amplification as a typical experimental setup for an evolutionary biotechnology application.*

INTRODUCTION

Optimizing macromolecules in accordance with the rules of natural evolution is a new strategy that has recently been introduced into modern biotechnology. This evolutionary biotechnology is based on applying the selection principle to populations of replicating molecules, viruses, microorganisms or cell populations that are subjected to externally controlled selection pressures (2,6,7). In this way, biological macromolecules evolve towards final products, which are optimized for a specific application, e.g., ligand binding (9,13,23) or catalytic function (1–3).

The classical experiments of "evolution in the test tube" by Spiegelman and coworkers (16,21) were based on the amplification of specific short sequences of RNAs using the enzyme *Q*β replicase. Recently other enzymatic amplification techniques have been introduced into molecular *in vitro* evolution: polymerase chain reaction (PCR) (e.g., References 1–3,23), 3SR (self-sustained sequence amplification) (5) and SDA (strand displacement amplification) (24).

The statistical analysis of evolutionary phenomena requires screening of large numbers of molecules in isolated compartments (7). Different species must be studied under identical reaction conditions, and, vice versa, the evolution of one species must be observed under different environmental constraints. Data obtained from these analyses can assist in the evaluation of new optimization strategies (20). To apply these statistical methods on evolutionary experiments with biological macromolecules utilizing amplification reactions like PCR (12,19), a machine for homogeneous and fast temperature cycling of many samples in parallel is needed.

Many automated systems have been developed that accomplish accurate temperature cycling in appropriate reaction vessels (e.g., References 11,18, 25). Some systems employ a robotic arm and a rack to move the sample tubes between water or oil baths. These systems generally ensure a uniform temperature distribution in the reaction vessels that is required for homogeneous reaction conditions. More common are programmable devices that produce different temperatures by heating and cooling a single metal block. However, these PCR machines do not always provide a uniform temperature distribution across the block, especially when cycling rapidly. Some commercially available temperature cyclers circumvent this problem by using a stirred water bath or oven at the expense of fast cycling (22).

Here we present an automated temperature cycler for evolutionary experiments with up to 960 parallel PCR samples of 10 – 40 µL in volume, utilizing three separate thermostating

aluminum blocks. A vacuum system ensures close contact between these blocks and the moved sample carrier. To deal with such a large number of samples and to avoid cross-contamination, it was necessary to develop new techniques of sample handling and processing. We therefore constructed special sealable and disposable plastic reaction vessels. All components of the apparatus were tested with RNA and DNA amplification reactions and a PCR serial transfer system modeling the technical requirements necessary for evolutionary experiments. Both critical requirements—the uniformity of temperature for all samples and rapid heating—are met, and the overall processing time is short.

MATERIALS AND METHODS

The main components of the PCR machine (Figure 1) are *(i)* plastic reaction vessels, each of which is a plastic sheet with 96 reaction vessels in a microplate format (see Figure 2A and Sealing of the Samples section below); *(ii)* a heat sealer to seal the PCR samples in the reaction vessels (see Figure 2B and Sealing of Samples); *(iii)* a liquid delivery system including two independent pipetting robotic arms to fill and empty the reaction vessels containing the samples; *(iv)* a transportation unit for moving the sample carrier (holding 10 plastic sheets with 96 reaction vessels each, i.e., a total of 960 samples) between the different pipetting and temperature stations; *(v)* three thermostats to perform fast temperature adjustments required for PCR and a freeze-storage device to store the reaction solutions for further analysis; and, *(vi)* a process control unit with several computers based on the VME (Versa Module Eurocard) data bus (a special computer system).

Sealing of the Samples

Sealed plastic sheets with reaction vessels were developed to ensure fast processing under identical conditions for each sample and to minimize evaporation and cross-contamination (Figure 2A). The reaction vessels are made from films extruded from Macrolon® (Kunststoffe Arthur Krüger, Hamburg, Germany); these proved to be chemically neutral in the test series. The bottom sheets (107 × 71 mm) with an initial thickness of 270 µm are placed on an aluminum mask containing the wells and then subjected to hot air (270°–300°C) from above. The reaction vessels are sealed after the wells have been filled with biochemical reaction solution. Sealing is achieved by welding a 100-µm-thick Macrolon cover foil to the sheet containing the reaction vessels by a heat sealer (Figure 2B). The finished and sealed reaction vessels have a volume of 40 µL and a wall thickness of 40 µm.

As an alternative sealing method, two commercially available ultrasonic wave generators (Model 900 M [20

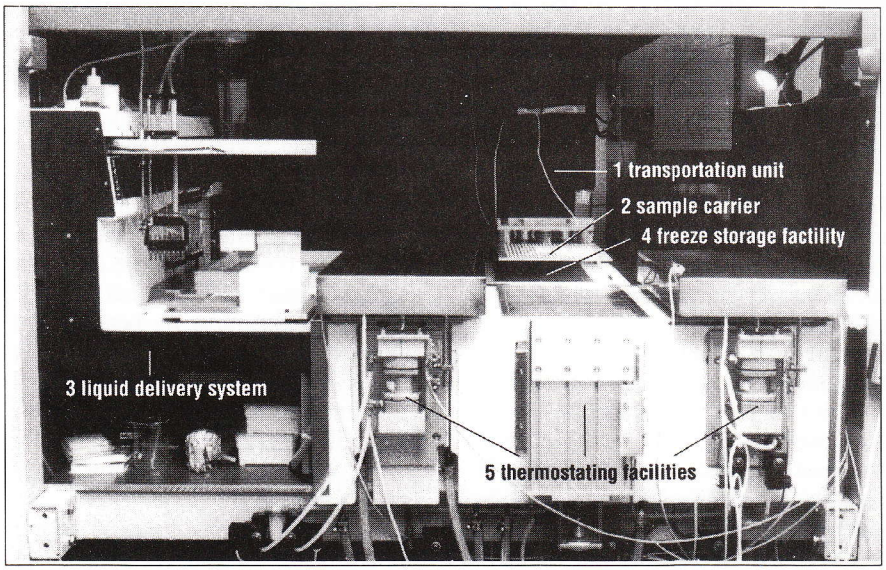

Figure 1. Scheme of the multichannel PCR machine. The transportation unit [1] moves the sample carrier [2] between the different pipetting [3] and temperature stations [4,5]. The liquid delivery system [3] includes two independent pipetting robot arms to fill and empty the plastic sheets containing the samples. The three thermostating facilities [5] required for the PCR and the freeze-storage facility [4] are shown. The aluminum cover of the central thermostat is removed. The sample carrier is machined with 960 wells in the shape of the reaction vessel cavities to accommodate up to 10 plastic sheets with 96 wells each.

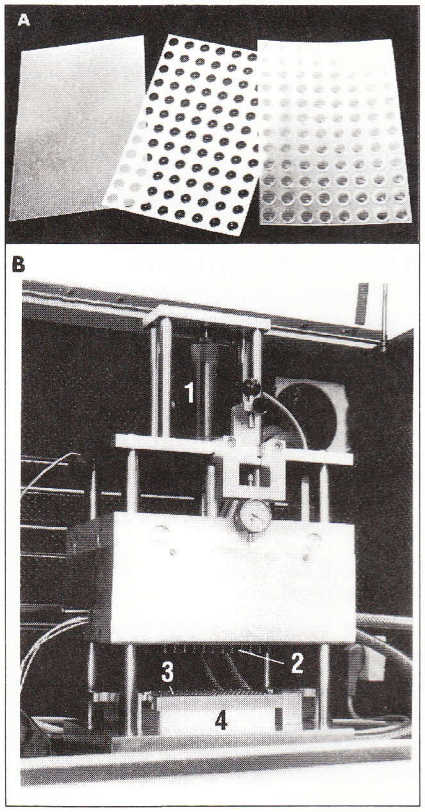

Figure 2. The plastic reaction vessels and the heat sealer. A) 96-reaction wells are contained on one plastic sheet. Left: cover sheet, middle: bottom sheet with wells, right: sealed plastic sheet. B) Photo of a sealing device for a plastic sheet with 96 wells. A stepping motor [1] drives the block with the heating cartridges [2] down onto a Teflon block [4] carrying the plastic sheets that are fixed by an aluminum mask [3]. After reaching the surface of the plastic sheets, the cartridges—at a temperature of 273°C—are slowly lowered a total of one millimeter in five steps thereby sealing the two sheets vacuum-tight within 10 s. The insertion of the sheets into the heat sealer is manually done on a separate bench. A patent application for this heat sealer has been filed (Reference 8).

KHz] and Model 947 M [40 KHz]; Branson, Hannover, Germany) were tested. By placing a sonotrode directly on top of the two sheets, the reaction vessels are sealed within several milliseconds.

Liquid Delivery System

A pipetting robot RSP 5052 with two arms (Zinsser Analytic; Tecan Robotics, Frankfurt, Germany) automatically delivers the samples to the reaction vessels using aerosol-resistant tips (ART) (Biozym, Hameln, Germany) on an arm with an 8-channel adaptor, filling eight vessels in parallel. To empty the reaction vessels, the second arm of the robot automatically punctures the cover film and then extracts the reaction solutions. Sterile cannulas with luer manus norm are used in order to minimize cross-contamination.

Transportation Unit and Sample Carrier

To guarantee fast temperature transitions together with convenient sample handling, we used an x, y, z - manipulator (CNC machine 82 V4; Föhrenbach, Löfflingen, Germany). It moves the sample carrier made of aluminum (444 × 260 × 5 mm) among the three different temperature stations required for the PCR. The sample carrier is machined with 960 wells in the shape of the reaction vessel cavities to accommodate up to 10 plastic sheets with 96 wells each.

Thermostating and Freeze Storage Facilities

All thermostats are constructed from large aluminum blocks (490 × 270 × 290 mm) to provide a high heat capacity. This ensures that their temperature remains constant even when the cooler or warmer sample carrier is placed on it. Furthermore, this guarantees temperature homogeneity across the aluminum block.

The desired temperatures are maintained by heating elements placed inside the blocks and by cooling with water. The temperature of the sample carrier is measured by a Pt 100 thermocouple probe (type M-FK, measurement based on the temperature dependency of a resistance; Heraeus, Hanau, Germany), directly affixed to the sample carrier with silicon glue. Homogeneity of static temperature across the sample carrier was experimentally determined to be 0.5°C. To measure temperature inside a water-filled well of a heat-sealed reaction vessel, a NiCrNi sensor (type GTF 300; measurement based on the Seebeck effect; Greisinger, Regenstauf, Germany) was inserted through the cover sheet, and the sheet was sealed with hot-melt glue (Henkel, Düsseldorf, Germany).

After moving the samples to the appropriate thermostats, the sample carrier is pressed onto the chosen station by a vacuum system integrated into each block. The high thermal conductivity of aluminum and the small thickness (5 mm) of the sample carrier account for a high heat transfer rate. To achieve a homogeneous and fast temperature adjustment for all 960 samples, an additional preheated aluminum cover is lowered onto the samples automatically after the sample carrier is positioned on the appropriate station.

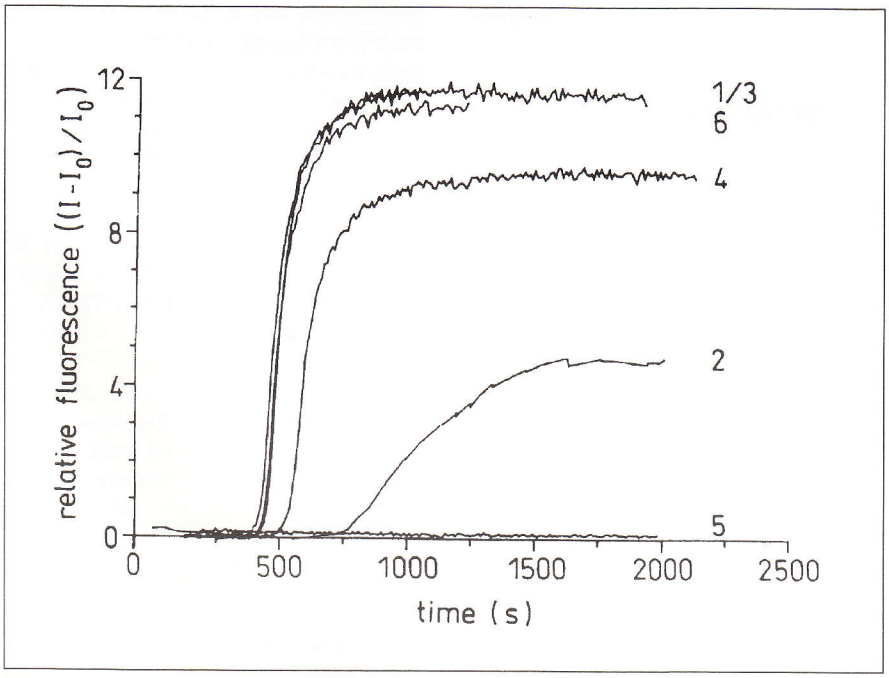

Figure 3. Fluorescence monitoring of RNA amplification by Qβ replicase after sealing the enzyme-containing reaction mixture and removing it from the reaction vessel. Sealing techniques and subsequent reaction conditions are described in Materials and Methods. Relative fluorescence signals are plotted vs. the reaction time of RNA replication. Curve 1: Reference (unsealed reaction mixture); curve 2: a liquid Qβ reaction mixture was heat sealed; curve 3: a frozen Qβ reaction mixture was heat-sealed; curve 4: the reaction mixture was liquid, but the block for the heat-sealing device was thermostated to -10°C, curve 5: a liquid reaction mixture was sealed with ultrasound; and curve 6: a frozen reaction mixture was sealed with ultrasound.

BIOCHEMICAL TESTS

Qβ System

To test the compatibility of the sealing procedures with the Qβ system, the reaction vessels were filled with 25-µL aliquots of reaction solution containing 50 mM Tris-HCl pH 7.5, 10% (vol/vol) glycerol, 10 mM dithiothreitol, 0.5 mM each of ATP, CTP, GTP and UTP, 1.0 µM ethidium bromide, 100 mM NaCl, 1 µM Qβ replicase and 1 nM of the self-replicating RNA MNV 11 (4). The reaction vessels were sealed as described above and immediately chilled on ice. After removing the samples with a sterile microsyringe, the contents of every five adjacent reaction wells were pooled to provide enough solution for fluorescence measurements in ultra-micro cuvettes with 100-µL volumes. RNA amplification by Qβ polymerase was monitored by measuring the fluorescence increase of ethidium bromide during amplification at 30°C in a thermostated fluorometer

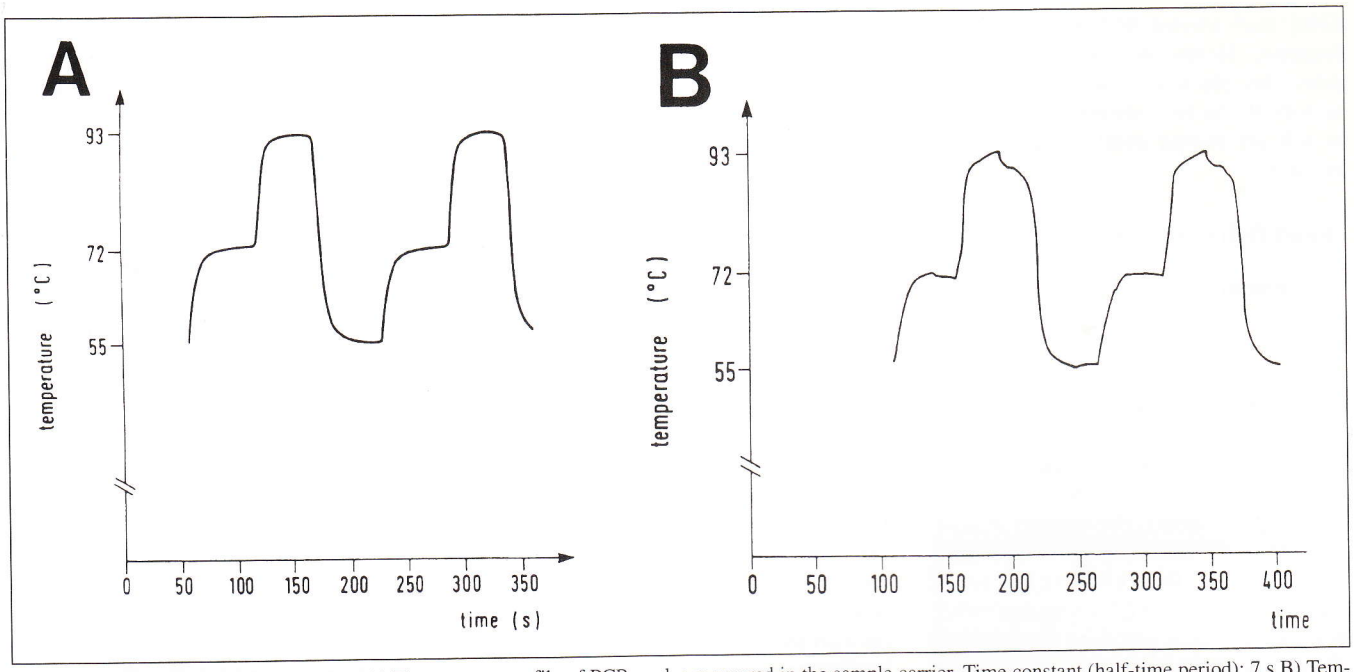

Figure 4. PCR cycling by the machine. A) Temperature profile of PCR cycles measured in the sample carrier. Time constant (half-time period): 7 s B) Temperature profile of PCR cycles measured in the solution inside a plastic reaction vessel placed onto the corner of the sample carrier. Time constant (half-time period): 8.5 s.

LS5B (Perkin-Elmer, Ueberlingen, Germany). Excitation wavelength was $\lambda = 514$ nm, emission wavelength was $\lambda = 600$ nm. RNA replication was initiated after 5-min preincubation by adding 1 µL of a 1 M $MgCl_2$ stock solution. A control reaction was performed using the same solution except that it was not sealed in plastic.

These experiments were repeated by sealing a stock solution of 4.2 µM MNV 11—RNA in water and amplifying the RNA with fresh $Q\beta$ replicase according to the protocol described above. All reaction mixtures were analyzed on a nondenaturing 11% polyacrylamide gel, which was photographed after ethidium bromide staining.

PCR Amplification

DNA template (Sequence: 5′-GGGATTTTTCCTTTTTCTCCCTCGCGTAAGCTAGCTACGCGAGGTGACCCCCCCCCGGGGGTTCTCCCCTATAGTGAGTCGTATTA-3′) and oligonucleotide primers (primer I: 5′-TAATACGACTCACTATAGGGGAGAACC-3′; primer II: 5′-GGGATTTTTCCTTTTTCTCCCT-3′) were synthesized on a Gene Assembler® Plus DNA Synthesizer (Pharmacia Biotech, Freiburg, Germany) and purified by denaturing polyacrylamide gel electrophoresis (PAGE). To test the different sealing techniques, 25-µL aliquots of PCR mixture containing 0.5 pM DNA template, 0.5 µM of each PCR primer, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM each of dATP, dCTP, dGTP

Table 1. Time Needed for the Different Pipetting Steps

Process	Filling of one plastic sheet (96 wells), 8-channel arm	Filling of 10 sheets (960 wells), 8-channel arm	Serial dilution (5 times), 8-channel arm	Filling of one plastic sheet (96 wells), 8-channel arm	Emptying 8 wells, 1-channel arm
No. of different solutions	1	1	1	2	8
Technique of dispensing	multi-dispensing, 1 × 8 tips	multi-dispensing, 10 × 8 tips	different positions, 5 × 8 tips	different positions, 2 × 8 tips	different positions, single cannulas
Time	2 min	20 min	6:30 min	3:35 min	2:30 min

The PCR process time for 34 temperature cycles of the described system is 1.33 h. The whole process time of the serial transfer PCR experiment (3 subsequent PCRs, 25 cycles each) including all automatic steps and sealing is 3.5 h. Insertion of the plastic sheet into the heat sealer and subsequent sealing is done manually on a separate bench and takes 2 min.

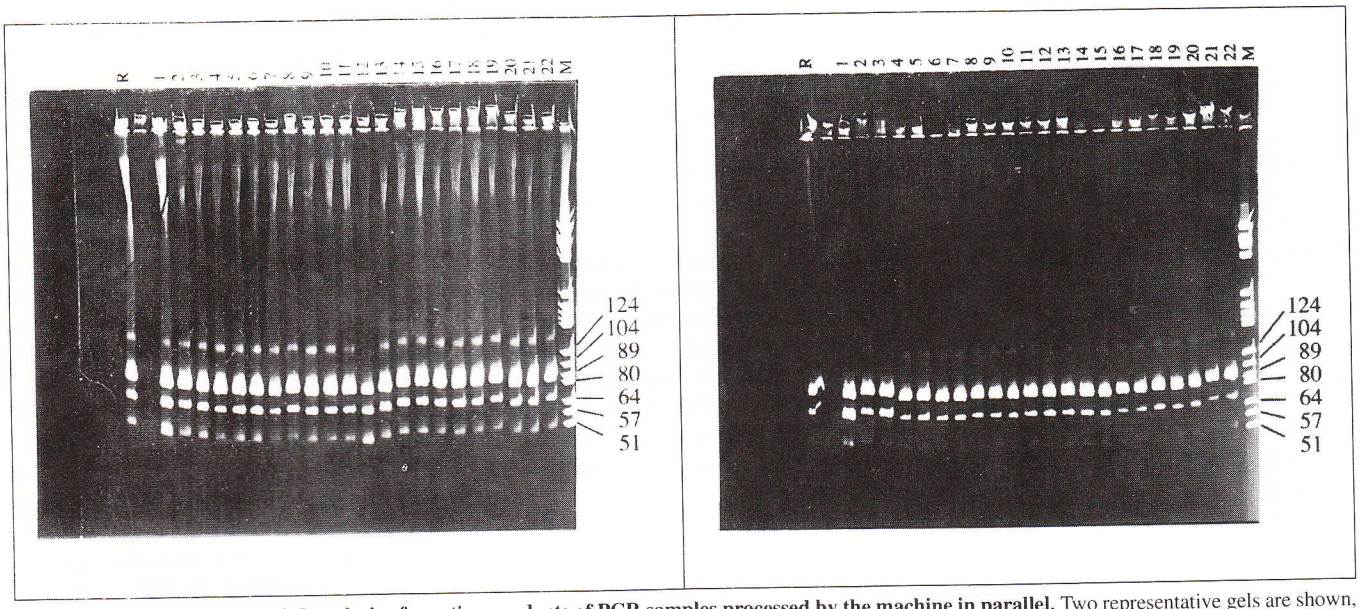

Figure 5. Non-denaturing PAGE analysis of reaction products of PCR samples processed by the machine in parallel. Two representative gels are shown, as described in Materials and Methods, with 44 individual samples (lanes 1–22) from all areas of sample carrier. Both gels contain a sample processed in a commercial TRIO-Thermocycler in lane R and 2 µL DNA marker V (Boehringer Mannheim, Mannheim, Germany) in lane M. Molecular weights are given in bp.

and dTTP and 0.04 U/µL *Taq* DNA Polymerase (Stratagene, Heidelberg, Germany) were distributed on the plastic sheets and subjected to the welding procedures described above. Twenty microliters were removed from each reaction chamber with a microsyringe and transferred to commercial 0.5-µL plastic tubes. An additional 20 µL of PCR mixture were not sealed and served as a control solution. Ten microliters of mineral oil were added to each PCR sample before subjecting them to 34 cycles of amplification. The following temperatures and reaction times were employed by a commerical TRIO-Thermocycler™ (Biometra, Göttingen, Germany): 30 s at 93°C, 30 s at 55°C, 30 s (7 min during the last cycle) at 72°C. Finally, they were stored at 10°C until they were analyzed by nondenaturing 11% PAGE.

Similar experiments were performed by sealing a stock solution of 1 pM of DNA template in water and subsequently subjecting it to a PCR with fresh primers and *Taq* DNA Polymerase, according to the protocol described above.

To test the uniform processing of parallel PCR samples by the robot described here, 96- × 30-µL volumes of the above-described PCR mixture, but with 90 template molecules per µL (0.15 fM) and 0.02 µg/µL of salmon sperm DNA (Sigma-Aldrich, Deisenhofen, Germany), were heat-sealed in the reaction vessels. The plastic sheet was cut by hand into pieces with 2 × 2 reaction chambers that were distributed uniformly over the whole area of the sample carrier. After a total of 45 temperature cycles as described above, 10 µL of each sample were removed for analysis by nondenaturing 10% PAGE.

Finally, to test the suitability of the multichannel PCR apparatus for evolutionary experiments, a serial transfer experiment was performed employing all the components of the machine. Forty-eight reaction chambers in four rows (every other row: rows B, D, F and H in the microplate format) of a plastic sheet were filled by the pipetting robot with the above-described PCR mixture but with 1.4 pM of DNA template and 0.02 µg/µL of salmon sperm DNA. The 48 remaining chambers in the rows in between (rows A, C, E and G) were likewise filled with reaction mixture excluding template. After heat-sealing the reaction vessels, the sheet was subjected to 25 temperature cycles on the sample carrier as described above. Subsequently, the pipetting robot removed 3.6 µL from the first reaction chamber of each row, diluted it in water 1:86000 times by five serial dilutions in a microplate and then mixed 15 µL of the final dilution with 15 µL of twice-concentrated PCR mixture excluding template, in each of the 12 chambers of the appropriate row in a fresh plastic sheet (so that the filling pattern with [in rows B, D, F and H] and without [in rows A, C, E and G] template was maintained). Again, 25 temperature cycles were performed after heat-sealing the reaction vessels, and the whole procedure of diluting the products and initiating a further round of PCR amplification by serial transfer to the appropriate row of a new plastic sheet was repeated. Volumes of 10 µL were removed from each individual reaction chamber of the three plastic sheets and analyzed by nondenaturing 10% PAGE.

RESULTS

The biological compatibility of different sealing techniques was tested with RNA-dependent RNA polymerase of $Q\beta$ phage that replicates a number of nonphysiological short RNA templates (4) and with *Taq* DNA Polymerase that amplifies DNA molecules in a subsequent PCR (12,19). Neither the thermostable *Taq* DNA Polymerase nor the nucleic acids were significantly af-

fected by the heat or ultrasound-sealing procedures (results not shown). However, the heterotetrameric and quite unstable $Q\beta$ replicase (14,15) showed a great loss of activity. This was evidenced by the lower increase in ethidium bromide fluorescence in a subsequent amplification reaction (Figure 3) and confirmed by gel electrophoresis. This inactivation is attributable to denaturation of $Q\beta$ replicase during the sealing procedure with heat (and not cooling the sample) or with ultrasound. We found, that the most reliable sealing technique to minimize the loss of activity of sensitive biological samples was sealing by heat on a Teflon® block with cooled or frozen samples (curve 3, Figure 3). Based on this technique, a parallel sealing device was constructed that can handle up to 96 samples on a single plastic sheet (Figure 2 and Materials and Methods).

Multiple thermostating blocks were used to obtain maximal cycling rates for a large number of samples. Instead of employing one block that must be heated and cooled repeatedly, a separate block exists for each desired temperature. Rapid changes among these temperatures can be achieved by transferring the samples among the different stations and pressing them onto the blocks with a vacuum system. The use of separate blocks also ensures spatial and temporal homogeneity of the different temperatures, whereas devices that heat and cool the samples in one metal block often show substantial differences in the temperature profiles of different samples when operating in rapid cycles (22).

Typically obtained PCR temperature profiles are given in Figure 4. Figure 4A shows a temperature profile measured in the sample carrier; Figure 4B shows a profile measured in the plastic reaction vessels. The temperature jump begins when the sample carrier is placed on the appropriate block and the vacuum system is switched on. The time required for a temperature adjustment of an aqueous solution in the reaction vessels from 55° to 72°C is 30 s, from 72° to 93°C is 30 s and decreasing the temperature from 93° to 55°C takes 45 s. In a dynamic measurement at different locations all over the sample carrier, we obtained an average time constant (half-time period) of 14.9 s (standard deviation of 5.3 s) in solution in comparison to 7 s (standard deviation of 1 s) measured in the sample carrier. A further reduction of the time constant (8.5 s in solution, standard deviation of 1.3 s) can be achieved by using heat-conducting grease between the reaction vessels and the sample carrier. This proved not to be necessary for the described PCR system.

The time constant for temperature changes is about 9 times larger when the vacuum system is not used. An arbitrary temperature profile between the two extremes can be simulated by periodically switching the vacuum system on and off to achieve the desired heat transfer rates. This flexibility is important because it is known that DNA amplification is critically dependent on temperature-time profiles (25).

Different profiles were tested for their efficiency and accuracy of amplification across the sample area. As a test system, we used a PCR with low template (0.15 fM) and high nontemplate (0.02 µg/µL) (salmon sperm) DNA as described in Materials and Methods. Employing the vacuum system, uniform products were found in an experiment with 96 separate samples of each 30-µL volume distributed uniformly over the whole sample carrier (Figure 5). An experiment with 960 ten-µL samples in 10 plastic sheets processed in parallel, which were analyzed by PAGE of each 12 pooled samples, showed equivalent results.

Finally, a serial transfer experiment was used to test all components of the device in an evolutionary type of process. A defined pattern of reaction

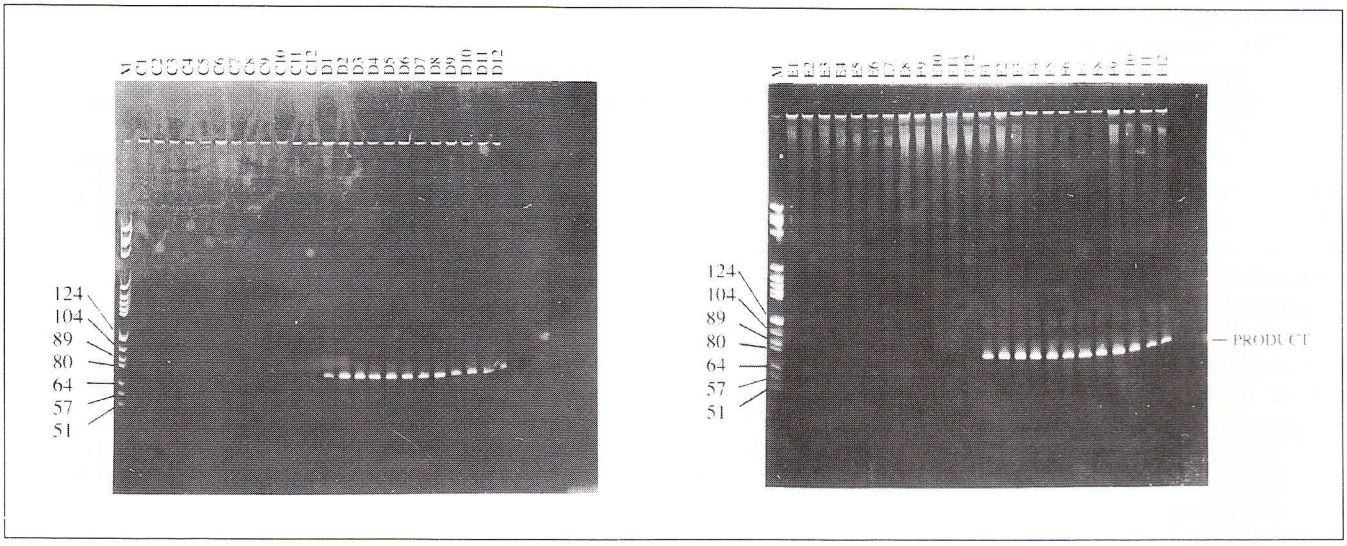

Figure 6. Non-denaturing PAGE analysis of twice serially transferred PCR products. Two representative gels of the products of the last of three subsequent PCRs are shown as described in Materials and Methods. Both gels contain 2 µL of DNA marker V in lane M. Molecular weights are given in bp. The left gel shows the products of wells C1 to C12 and D1 to D12; the right gel shows the products of wells E1 to E12 and F1 to F12 after two serial transfers. Rows C and E of the microplate-formatted plastic sheet show no products, while rows D and F do show products, indicating that the initial filling pattern is maintained throughout the serial transfer experiment without cross-contamination.

chambers was filled by the pipetting robot with PCR mixture including template and neighboring reaction chambers with PCR mixture excluding template (see Materials and Methods). After two serial transfers including the dilution of reaction products and maintaining the filling pattern in the plastic sheet, we found that all samples were correctly processed without cross-contamination (Figure 6). Table 1 shows the times necessary for the different processing steps.

DISCUSSION

Our experiments with PCR mixtures in 96 and up to 960 sample wells show that our device is suitable for parallel processing of this biological reaction under reproducible and homogeneous conditions. In contrast to other approaches (10,17), it is optimized for evolutionary types of experiments. This was shown by applying the serial transfer method (21,24) to a PCR. Similar experiments simulating conditions for Darwinian evolution (5,9,24) are now possible with up to 960 samples that can be processed in parallel.

We also showed that solutions containing $Q\beta$ RNA polymerase can be sealed without loss of activity so that this isothermal amplification system can be employed in our machine as well. As demonstrated previously (4,16), RNA molecules replicated by $Q\beta$ polymerase can be used as a model system for studying evolution at the molecular level. Moreover, all components of our system (the plastic materials, the setup with three heating blocks and a sample carrier that is accessible from above) are suitable for performing fluorescence measurements directly in the reaction chambers. Experiments are in progress where both PCR and replication by the $Q\beta$ enzyme are monitored using fluorescent dyes to follow the evolution of the nucleic acid molecules directly *in vitro*.

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