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On-line polymerase chain reaction (PCR) monitoring

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Abstract

In this short note, we present the results of a case study for monitoring the whole polymerase chain reaction (PCR) process (all steps) with a glass fiber fluorometer that was described in a former publication. To utilize this fluorometer, which was originally constructed for a PCR machine with three thermostatting devices, a new thermostatting device has been developed: the glass fiber matrix is integrated into the thermostatting device, while the PCR samples are heated and cooled. The device is able to monitor all samples throughout all stages of PCR with the help of an intercalating dye. This approach also permits one to choose arbitrarily different cooling and heating rates. © 1998 Elsevier Science B.V. All rights reserved.

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The polymerase chain reaction (PCR) is the most widely used in vitro amplification technique for nucleic acids. Alternating temperature steps are required for denaturing double-stranded DNA (dsDNA) and for annealing short primers to their target sequences for subsequent elongation [1]. A lot of large-scale PCR machines for processing PCR samples in parallel, mostly based on three temperature positions, have been developed [2,3]. Higuchi et al. [4,5] constructed a device for monitoring the signal during all three temperature steps of the complete amplification process. Different primer and template concentrations could be detected. Here, we describe an extension of this technology to monitor 96 channels in parallel with an improved signal-to-noise ratio.

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Previously, a large-scale automaton for processing large arrays of samples and for monitoring them on-line was presented; all details concerning the amplification technique have been described [6–8]. In this automaton, a glass fiber matrix was integrated into one of the three temperature blocks. Thus, PCR could be recorded at only one of the three temperature steps (e.g. at the elongation step). Due to this coarse recording window, limitations in sensitivity were inevitable.

We have significantly improved the technique by constructing a device where the sample carrier (carrying up to 96 samples) actively controls the sample temperature, rather than moving the samples to different temperature reservoirs. Thus, all 96 solutions can be monitored by the detection unit throughout the amplification process, allowing images to be recorded continuously using the technique described by Schober et al. [7]. As before, the nucleic acid concentrations are quantitated by the fluorescence of the intercalating dye, ethidium bromide (EtBr). For this case study, only 96 of all the possible 960 channels of the fluorometer were used.

The principle of operation of this apparatus for on-line monitoring PCR is shown in Fig. 1. The central part is a newly designed thermostatting device for performing rapid temperature variations, as required for the PCR (Fig. 2); this is coupled to an optical detection unit comprising a glass fiber fluorometer (excitation wavelength, 514 nm; fluorescence signal, 600 nm) with, in our case study, 96 excitation and 96 emission fibers and a CCD camera with on-line image processing software running on a VME data bus computer. Samples for PCR were filled into plastic reaction vessels (a plastic sheet with 96 reaction vessels) and sealed, using heat, with a cover foil.

Regulation of the temperatures was more complex than in the previously described thermostatting variant that was based on three different temperature stations. The new device carries a sample holder with 13 integrated heat cartridges. Because it is difficult to achieve homogeneous temperature distributions in single-block devices when the temperature must be changed rapidly, we distributed the process of heating by using 13 cartridges (Helios, Neuenrade, Germany) positioned only 1 mm apart from each other (Fig. 2).

The spatial temperature deviations during the temperature change vary by less than

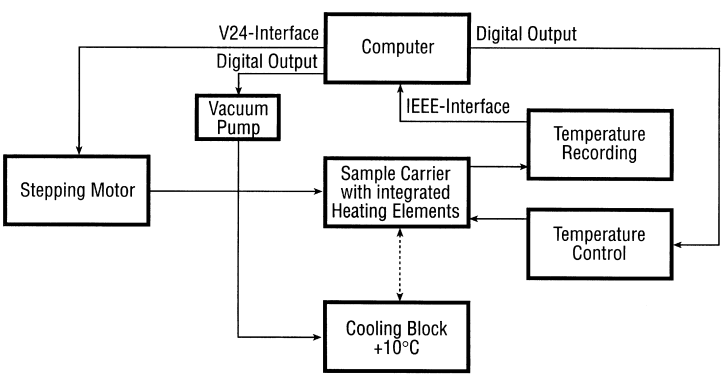

Fig. 1. Principles of operation of the apparatus for on-line monitoring PCR.

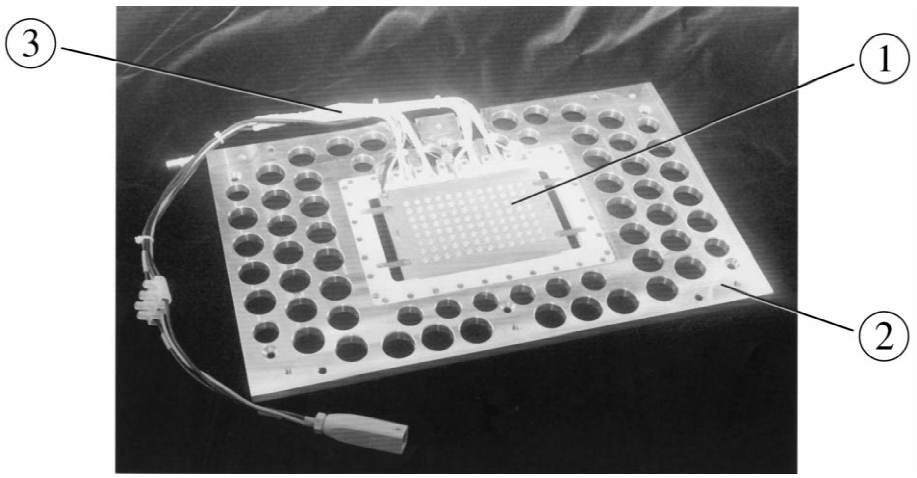

Fig. 2. Sample carrier. A plastic sheet (not shown) containing the PCR solutions in its 96 wells was centrally fastened to the sample carrier (1). The sample holder was thermally insulated from the outer frame (2). The cables for the electric supply of the heating cartridges are visible (3). This design allows high heat transfer rates and, thus, high processing speeds. After loading with the samples, the sample carrier was placed under the detection unit for on-line monitoring of the amplification process. The cooling block is not shown.

0.5°C (not shown). Theoretically, the rate of the temperature change is determined only by the power, *P*, of the heat cartridges (2.1 kW), the mass, *m*, of the sample holder (0.378 kg) and the specific heat capacity, c_p, of the material used, aluminum (940 J/(kg · K)). The cooling step (for primer annealing) was performed by lowering the sample carrier (together with the flexible glass fiber portion of the detection optics) using a stepping motor and pressing it onto an aluminum cooling block with a vacuum. As soon as the lower annealing temperature (58°C) was reached, the sample carrier was raised from the cooling block.

The large aluminum cooling block ($490 \times 270 \times 290$ mm) has a high heat capacity and dissipates the heat rapidly. The desired temperature of the cooling block was maintained by water cooling in addition to the computer-controlled heating by the cartridges. In this way, rapid changes between the desired temperatures can be achieved. The theoretically possible heating rate of this device is then obtained using the equation $\Delta t = (mc_p\ \Delta T)/P$; for a temperature jump from 58°C (T_2) to 92°C (T_1), this is approximately 5.6 s. The theoretically possible cooling rate for the same temperature jump is controlled by the thermal resistance, *R*, the capacity, *C*, of the carrier and, the temperature, T_r, of the cooling basin, which is especially important for this device. In a first order approximation, assuming that the temperature decay behaves like an exponential function, the necessary reservoir temperature, T_r, can be calculated for a given cooling time and the desired temperature T_2 with $T_r = (T_2 - T_1 \exp(-t/RC))/(1 - \exp(-t/RC))$. For example, for a cooling rate of 5.6 s, a reservoir temperature of approximately 13°C is needed.

The planar geometry of our device is required by the necessity for processing and

analyzing samples in parallel; this planarity demands a non-orthogonal arrangement of the excitation light beam and the fluorescence detection, which was realized by the glass fiber fluorometer described by Schober et al. [7].

Experiments with different primer concentrations were performed in order to test whether this PCR monitoring apparatus can detect differences in PCR amplification efficiency. Reaction chambers in a plastic sheet were filled with 30 μl of the PCR solution, including 3 μM EtBr for detecting the amplification signal. Some reaction chambers were filled with only 3 μM EtBr in a buffer solution, and some chambers remained empty, to serve as blank signals. As a target sequence for amplification, we chose a sequence of the *Tetrahymena* group I intron (434 bp). The temperature protocol of this reaction was 31 cycles at 92°C for 80 s, at 58°C for 115 s, and at 73°C for 115 s.

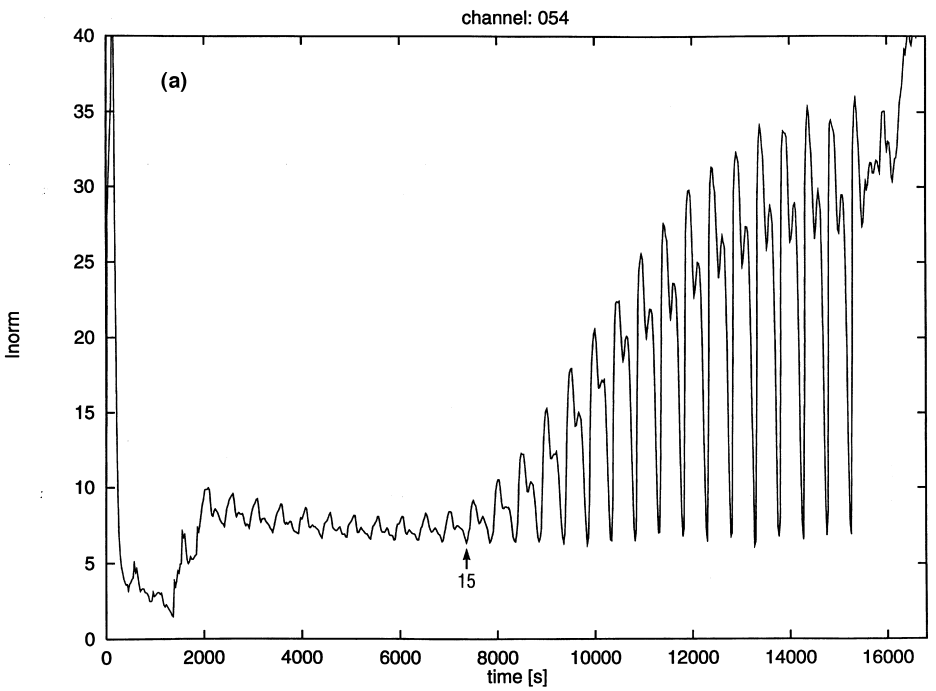

Fig. 3. Plot of the normalized fluorescence signal over time for the PCR process. (a) Primer (1 μM), defining the upper bounds of the final dsDNA product concentration. Superimposed on the sigmoidal shape of the overall amplification process is an oscillation that is synchronous to the temperature cycles: the fluorescence intensity is minimal at the melting temperature, increases to a maximum at the annealing temperature, decreases again at the beginning of the elongation temperature and, finally, has a minor peak at the end of the elongation temperature [see (c) also]. (b) A primer concentration of 0.05 μM. A later onset (cycle 19 as opposed to cycle 15) and a decreased absolute value of the signal are observed due to a smaller amount of the dsDNA product. The sharp signal increase at the end of the reaction is due to the final lowering of the sample carrier onto the cooling block. This cooling step (down to 10°C) enhances the ethidium bromide signal. (c) The lower curve (dashed) shows the normalized fluorescence signal of one sample in the saturation phase from cycle 25 to cycle 29. The upper curve (solid) shows the temperature profile in the sample carrier, which was measured simultaneously with the fluorescence signal.

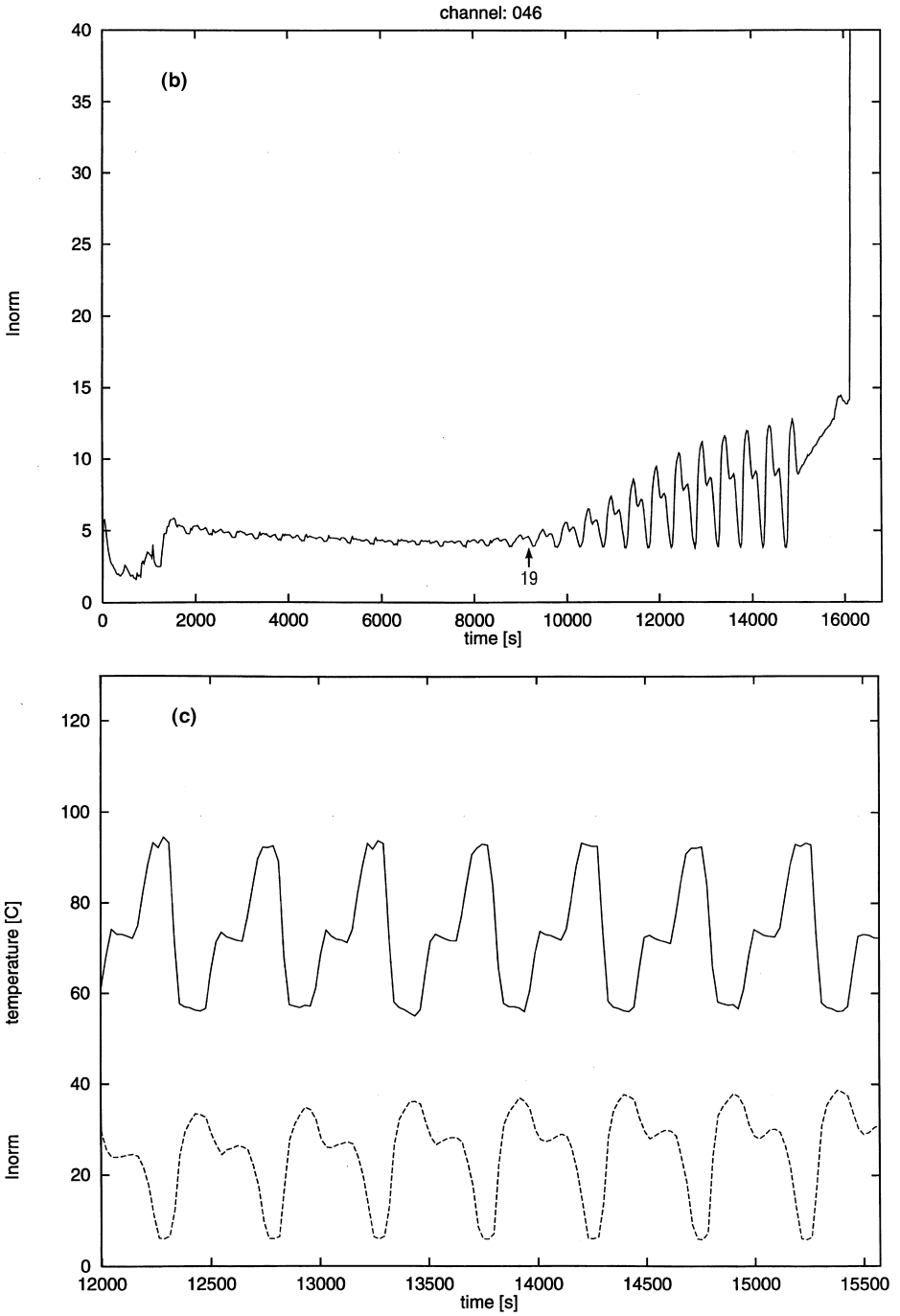

Fig. 3. (*continued*)

Reaction conditions for PCR had been optimized and are described by Schober et al. [7]. The temperature protocol was 31 cycles at 92°C for 80 s, at 58°C for 115 s, and at 73°C for 115 s. The time required for every cycle was 540 s.

The time course of the EtBr fluorescence with different samples is shown in Fig. 3. The empty chambers, as well as the chambers containing 3 μM EtBr in a buffer solution, did not show any significant signal above the noise threshold (not shown). Reaction chambers filled with the product of a previous amplification reaction exhibited signals with the periodic behavior of the amplification reaction without an overall increase of the signal. The periodic behavior of the amplification reactions in Fig. 3 can be attributed to the different fluorescence efficiencies of dsDNA–EtBr complexes at different temperatures. At its melting temperature (i.e. 92°C), the concentration of dsDNA is negligible and most of the EtBr is unbound. At the annealing temperature (i.e. 58°C), formation of the DNA complex is favored and a high fluorescence ensues due to a reduced quenching of the fluorophore. At the elongation temperature (i.e. 73°C), an intermediate fluorescence signal was observed that slightly increased over time and can probably be attributed to the replication process; while the polymerase is processing the single-stranded DNA in order to produce dsDNA, more EtBr can be bound in fluorescent complexes and the signal increases. Using standards, the observed relative fluorescence increase can be correlated principally with the PCR yield for any given PCR reaction.

The temperature principle described in this note can be adapted for larger sample carriers as described by Schober et al. [7].

Equipment for the on-line monitoring of specific DNA amplification by PCR will lead to complete control over multiple samples in parallel at any time during the amplification process. These features enable one to fully automate the process of analysis as, for example, is required in clinical diagnosis.

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