

## Articles

## Continuous Segmented-Flow Polymerase Chain Reaction for High-Throughput Miniaturized DNA Amplification

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A continuous segmented-flow method for sequential DNA amplification is described in order to provide a basis for high-throughput genetic analysis. The approach allows an immediate distinction between amplified and nonamplified products. A mixture of sample and reagents are loaded in the form of small segments one after another in a 15-m-long narrow-bore Teflon tube, coiled such as to be repeatedly exposed to three different temperature zones. After having passed the heated zones, the samples are mixed with an intercalating dye by flow injection and sequentially detected on-line by laser-induced fluorescence. The aqueous samples travel as separate segments in a continuous flow of an immiscible, organic liquid. Perfluorodecalin was shown to be particularly suitable due to its hydrophobicity and inert properties. To reduce carryover between samples, an intermediate water plug between two consecutive samples was required. Selected regions from human genomic DNA were successfully amplified in 300-nL volumes after 30 passes through the heated zones. The total reaction time was ~45 min, and the detection interval between individual samples was 1 min. Automation and the possibility to further reduce sample volumes, as well as to employ many reaction columns simultaneously, should provide a platform for an extremely high throughput.

The polymerase chain reaction (PCR) since its introduction<sup>1</sup> has revolutionized molecular biology and the way of studying, discovering, and understanding fundamental aspects of life. A specific DNA sequence is amplified by an enzymatic reaction using repetitive cycling between three temperatures. The product of the reaction can be diagnostic for the presence of genetic diseases

and for the presence of a specific bacterium or virus in a tissue or blood sample and can be used in many other genetic tests, e.g. forensic investigations.<sup>2</sup>

Genetic analysis by conventional methods, i.e., direct sequencing, is still a time-consuming, manually intensive, and costly procedure, although significant progress has recently been achieved in this field. PCR-based techniques such as denaturing high-performance liquid chromatography (DHPLC),<sup>3</sup> double gradient-denaturant gradient gel electrophoresis (DG-DGGE) on slab gel,<sup>4–6</sup> and single-strand conformation polymorphism by capillary electrophoresis<sup>7–9</sup> offer sufficient robustness for the screening of unknown single-nucleotide polymorphisms (SNPs), limiting the necessity to make use of direct sequencing to only those fragments of DNA where mutations have been found. The number of known SNPs in the human genome is now rapidly expanding. Recently, a map of 1.42 million SNPs was reported.<sup>10</sup> This has dramatically increased the need for high-throughput methods. A number of assaying technologies have become available, either based on hybridization, in the format of microarrays<sup>11</sup> for example, or based on synthesis, that is, covalent bond formation. Two major

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methods are Pyrosequencing<sup>12</sup> and minisequencing.<sup>13</sup> We have recently shown<sup>14</sup> how minisequencing, combined with capillary electrophoresis, can be transformed in a high-throughput method for assaying known polymorphisms. Also, the use of MALDI-TOF MS has been shown to be a powerful candidate for high-throughput SNP analysis.<sup>15</sup>

PCR can also be used in a more direct way. Valuable diagnostic information is obtained from the outcome of a qualitative PCR assay, that is, whether the PCR has generated an amplification product or has not led to any amplification. A straightforward application is, as mentioned above, the determination of the presence of foreign DNA, such as that of a virus or bacterium, in a tissue or blood sample. Moreover, with a modification of the PCR protocol, DNA genotyping seems possible directly from blood with no need to extract or purify the DNA.<sup>16</sup> Another application is the screening procedure to identify a desired gene from a genomic or complementary DNA library. It is also possible to assay SNPs by PCR only. There is a method, called the amplification refractory mutation system (ARMS),<sup>17</sup> based on the fact that the 3'-nucleotide of a PCR primer is critical for the success of the amplification. If one of the primers is designed so that this last nucleotide falls right at the site of the gene where a mutation occurs, the presence of the mutation will cause failure of the PCR and no amplification will be observed. To confirm the analysis, a second PCR with primers specific for the mutated sequence must be set up. The use of universal TaqMan probes<sup>18</sup> can be also considered as another attractive approach in this direction.

All these applications require carrying out a large number of PCRs, and enhancing the throughput of this methodology would have a significant impact in molecular biology and genome-based techniques. At present, the amplification rate depends on instrumental limitations, mainly thermal hysteresis, and not on the reaction kinetics. The samples are repeatedly subjected to the three different required temperatures and this might take as long as 2–4 h with the conventional block array devices, due to a large thermal mass and associated slow heating and cooling rates. High thermal cycling rates and efficient amplification are achieved by systems using glass capillaries containing the PCR mixture, where air streams are used for heating and cooling.<sup>19</sup> Swerdlow et al. showed an interesting automated system where they coupled thermal cycling, purification, in-line loading, and analysis by capillary electrophoresis in a single instrument.<sup>20</sup> Several other

groups have reported high cycling rates for PCR with various designs of micromachined heating chambers also coupled to electrophoretic analysis.<sup>21</sup> PCR devices using microfluidics have been fabricated in silicon,<sup>22–26</sup> glass,<sup>27–29</sup> and silicon glass hybrids.<sup>30</sup>

An additional way to enhance the PCR throughput is by multiplexing, where several reactions are carried out simultaneously in one mixture. However, there are a number of disadvantages, which are related to cross-interactions and competing chemistries.<sup>31,32</sup> Optimization of the design of primers is critical, to avoid undesired annealing, failure of amplification, or both. Moreover, differential amplification is usually observed, leading to uneven product yields, and adjusting the relative concentration of primers does often not solve this problem. Therefore the number of parallel reactions, which can be carried out by multiplex PCR, as well as the general usefulness of this approach, is limited. Thus, it is clear that limited throughput of PCR is still a bottleneck in applied genomics and that there is a great need for improved technologies.

All systems, which are referred to above, are based on static reactors, i.e., systems where the reaction occurs in a confined space, which is brought to different temperatures in a repetitive sequence. An alternative interesting principle is the dynamic reactor, where a continuous flow of samples and reagents is passed through three zones, which are kept at constant temperatures optimized for denaturation, annealing, and elongation of the DNA fragments. The principle was first described by Nakano et al.,<sup>33</sup> while Kopp et al.<sup>34</sup> as well as Poser and co-workers<sup>35</sup> showed the benefits of utilizing chip-based formats.

One of the features of flow-through reactors is that a continuous serial sequence of reaction mixtures can be performed, while problems related to thermal lagging are minimized. Also, the simplicity of the concept is very attractive. However, carryover and cross-contamination between consecutive samples becomes a major issue.<sup>35</sup>

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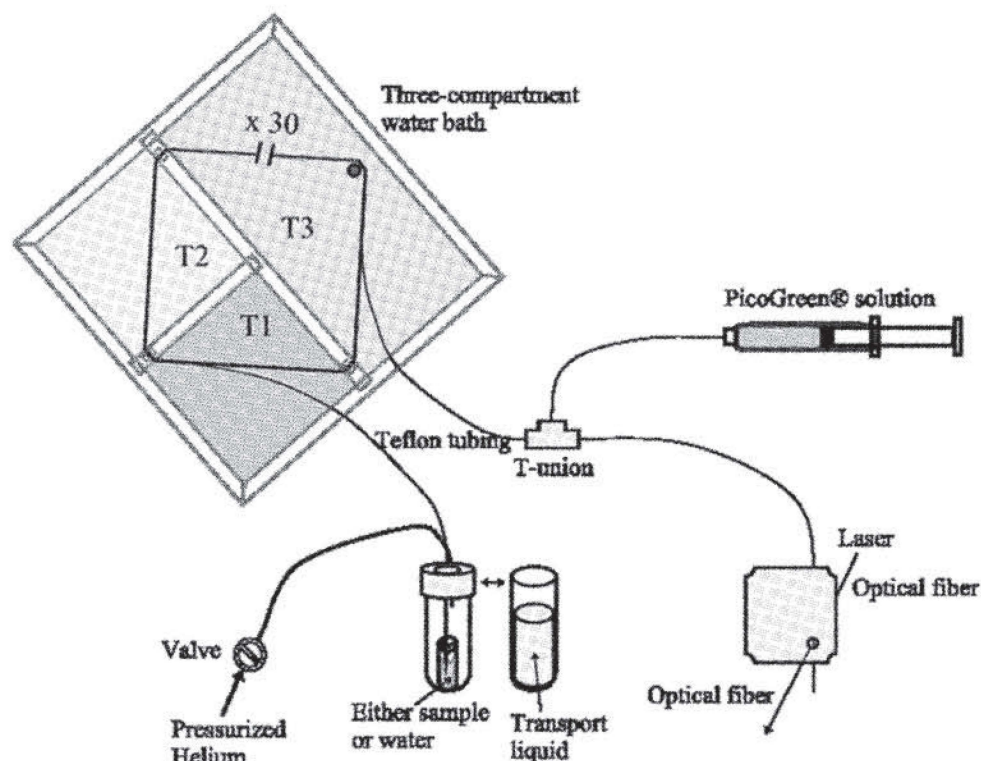


Figure 1. Schematic of the experimental setup for segmented continuous-flow PCR. After alternative manual injection of sample plugs and loading of carrier fluid plugs in a Teflon capillary tube, via pressurized vials, the samples are repeatedly passed through the three heated zones, by a continuous flow of the carrier fluid. Finally, the samples are mixed with PicoGreen dye and amplification is detected by laser-induced fluorescence.

In this article, we present a flow reactor concept, where the aqueous sample mixtures are separated from each other by an immiscible organic fluid. In this way, a very large train of individual small samples can be transported through the reactor, while carryover can be minimized. Detection of the amplified DNA template is performed on-line by laser-induced fluorescence after mixing with a suitable intercalating dye.

## EXPERIMENTAL SECTION

**Amplification Device.** A schematic of our setup is shown in Figure 1. A three-compartment device in polycarbonate and with a wall thickness of 8 mm was constructed. The reaction channel is a Teflon tube (200/400  $\mu\text{m}$  i.d./o.d.; Cole-Parmer, Vernon Hills, IL). A series of holes 0.5 mm in diameter are drilled in vertical array in each compartment wall, the thickness of which is locally reduced to 3 mm. In this way, the Teflon tubing can be repeatedly passed through the three compartments as many times as the desired number of PCR cycles. The length of the tubing in each compartment is proportional to the desired time ratio for the three PCR steps. Each compartment is filled with water. The temperature in each compartment is independently controlled by means of individual heating coils and Pt-100 sensors (TemFlow, Stockholm, Sweden) dipped into the water and connected to proportional, integrating, and derivatizing (PID) regulators (CAL Controls Inc, Libertyville, IL) while a magnetic bar agitates to keep the temperature homogeneous.

**PCR.** The tube, 15-m length from inlet to outlet, was passed 30 times through the three temperature zones, thus corresponding to 30 PCR cycles. The tube length per cycle was 44 cm with a

ratio 1:1:2 for the three steps: hence, 11 cm for the denaturing step (33 cm for the first cycle), 11 cm for the annealing step, and 22 cm for the elongation step (66 cm for the last cycle).

As DNA source, genomic human placenta DNA (Sigma-Aldrich, St. Louis, MO) was utilized at a concentration of 5 ng/ $\mu\text{L}$ . A 79-bp region of the  $\alpha$ -1-antitrypsin (A1AT) gene and a 148-bp region of the cystic fibrosis transmembrane conductance regulator (CFTR) gene were used as targets for amplification. Primers (purchased from Thermo Hybaid, Ulm, Germany), used at a concentration of 0.4  $\mu\text{M}$  each, were as follows: 5'-TGCAT-AAGGCTGTGCTGACC-3' and 5'-AGACATGGGTATGGCCTCT-3' for A1AT and 5'-ACTGGAGCCTTCAGAGGGTAAAAT-3' and 5'-ATGCTTTGATGACGCTTCTG-3' for CFTR. AmpliTaq polymerase (Sigma-Aldrich) was used at 0.02 unit/ per reaction plug (300 nL, corresponding to a sample plug length of  $\sim$ 1 cm) while the concentration of each dNTP (Sigma-Aldrich) in the reaction plugs was 200  $\mu\text{M}$ .

The temperatures in the three compartments were set as follows: denaturation at 94  $^{\circ}\text{C}$ , annealing at 54  $^{\circ}\text{C}$  and elongation at 72  $^{\circ}\text{C}$ . The flow rate in the tubing determines the residence time in each zone. The experiments were performed with a time frame of 90 s/cycle. Two negative controls were also used in the experiments: one contained all the reagents at the same concentration as for the normal reaction mixtures, however, without DNA template; another contained the DNA template but no DNA polymerase. All the reaction mixtures were freshly prepared in volumes of 15  $\mu\text{L}$  on a daily basis and kept at 0  $^{\circ}\text{C}$  before use.



**Sample Loading, Transport, and Detection.** A hydrocarbon, *n*-decane (KEBO AB, Stockholm, Sweden), and a fluorocarbon, perfluorodecalin (Chemtronica, Stockholm, Sweden), were tested and used as transport liquids. Pressurized helium was used to load the reaction mixtures and force their transport through the tubing. Each reaction mixture (15  $\mu$ L) was kept in a disposable vial to be introduced into a larger glass vial. The latter was closed by a screw cap equipped with a rubber septum. The tubing, and the capillary through which pressure was applied, were passed through the septum. The flow injection procedure was performed manually by turning off the valve, opening the large vial, and exchanging the disposable vial. After each injection, the cap with the tubing in place was switched onto a second vial, filled with carrier fluid. Typically, using the fluorocarbon as transport fluid, injection during 2 s at 30 psi corresponded to a sample plug of 1 cm and a volume of 300 nL. Using the less viscous *n*-decane for transport, a lower pressure (8 psi) was sufficient to inject the same volume and obtain the same cycling rate. The distance between two consecutive samples separated by organic liquid was  $\sim$ 30 cm (corresponding to a time space of 1 min). To minimize carryover, 1- $\mu$ L water plugs (corresponding to  $\sim$ 3-cm length) were introduced between samples while maintaining the 30-cm distance between samples. In experiments where the flow speed was doubled, the distance between samples was  $\sim$ 20 cm. After the last PCR cycle, the sample plugs are mixed with a continuously flowing solution of the intercalating dye PicoGreen (stock solution in DMSO; Molecular Probes Inc., Eugene, OR) diluted 1:200 in water. The dye solutions were introduced into the main stream through a T-union from the Teflon needle of a 1-mL Hamilton glass syringe (all the three pieces purchased from Scantec Lab AB, Stockholm, Sweden). A syringe pump (Cole Palmer, Vernon Hills, IL) provided a flow rate of 0.6 mL/h. The outlet of the reaction tubing was mounted into the cartridge of a Beckman P/ACE System 5500 capillary electrophoresis instrument equipped with an optical fiber-based laser-induced fluorescence (LIF) accessory.

**Safety Considerations.** PicoGreen should be treated as a potential mutagen and handled with appropriate care. The DMSO stock solution should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues. It is recommended to use double gloves when the stock solution is handled. Solutions of the reagent should be poured through activated charcoal before disposal. The charcoal must be then incinerated to destroy the dye.

## RESULTS AND DISCUSSION

The flow reactor principle is basically an attractive concept for PCR. The three static temperature zones eliminate the need for temperature cycling and minimize associated thermal hysteresis. Thus, the principle offers the potential for extremely fast DNA amplification. Also, the simplicity of the setup, compared to complex micromachined structures, is an attractive feature. However, some limiting factors need to be considered. The change in temperature of the reaction mixture, which passes from one zone to the next, is not instantaneous; some time is needed to reach each new temperature level. This time is dependent on the flow rate and the thermal conductivity of the transport tube and the reaction liquid. Thus, the use of thin-walled metal tubes or chip-based structures in silicon would be advantageous. Minia-

turization of the fluidic system is beneficial in two ways: it enhances the speed of thermal equilibration of the reaction mixture, thus allowing increased flow velocities and faster PCR. Also, analyte volumes are reduced, thereby decreasing the consumption of polymerase and reagents, while concentrations of these components can be maintained at an optimal level.

Earlier studies in flow-based PCR<sup>34</sup> have operated in a continuous mode, where the entire fluidic structure was engaged for single sample amplification. Subsequent samples were injected and amplified after rinsing the structure between each sample with an optimized buffer solution, and under these conditions (including a surface modification), problems with residual carryover were not experienced.

In the present work, another concept of flow injection is utilized, which is well known in other areas of FIA, e.g., in flow extraction.<sup>36,37</sup> The carrier liquid is a hydrophobic, inert fluid, immiscible with water, in which small volumes of the reaction mixtures are injected at short intervals. This results in a segmented flow, where each sample is an entity, well separated from the adjacent ones. An important condition is that the surface of the fluidic channel is also hydrophobic, otherwise surface wetting and film formation would cause a severe cross-contamination. In principle, air segmentation could be employed, but adverse effects, related to the compressibility of air, flow speed limitations due to droplet disintegration, and other effects that we discussed earlier,<sup>38</sup> make this a less attractive approach.

The choice of the hydrophobic transport liquid is also important. Preferably, the viscosity of the liquid should be low, to avoid large pressure drops, while the liquid should not be miscible with water and should have a boiling point higher than 100 °C.

In a first series of experiments, we made efforts to optimize the reaction and tested to which extent carryover occurs. Two control samples ( $C_1$ ), containing no DNA template but primers and all other reagents necessary for PCR, were placed in segments around a normal sample, which was expected to show amplification. In the absence of cross-contamination, the second  $C_1$  will not contain any amplified DNA. This can be verified by the intensities of the background signals for the two  $C_1$ s, which then should be similar. Thin-walled PTFE tubing without any wall treatment and with an inner diameter of 200  $\mu$ m was employed, while *n*-decane (boiling point 174 °C) was used as transport liquid. The 79-bp sequence of the A1AT gene ( $\alpha$ ) was utilized as target. To allow fluorescence detection of the amplified material, PicoGreen was added to the sample plugs by means of flow injection via a T-union. Fluorescence detection can be performed in different ways. For the reported experiments, we utilized a simple arrangement with optical fibers from a commercial CE instrument. This provided adequate sensitivity.

The results are shown in Figure 2A, which shows that successful amplification is achieved. However, a substantial carryover also occurs, even when the time frame between two consecutive samples is as much as 1 min. The cross-contamination could also be visually observed in a model experiment, where an

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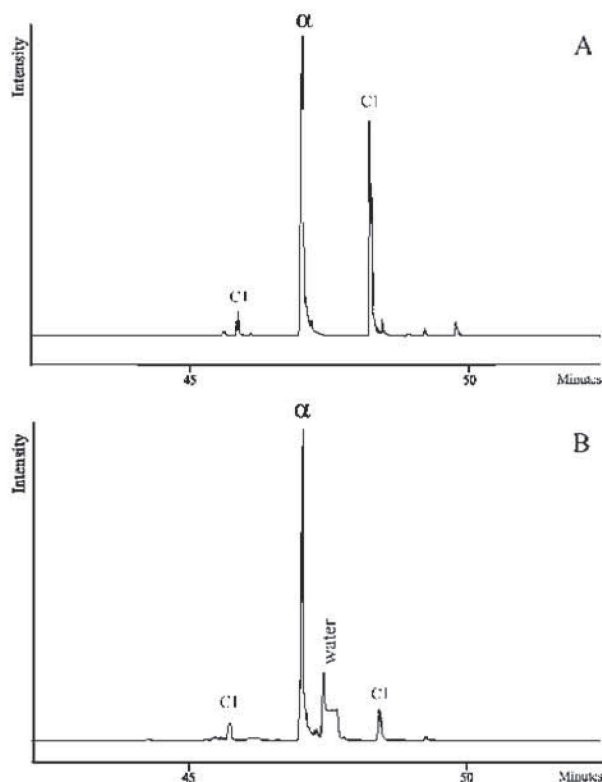


Figure 2. (A) PCR results using *n*-decane as transport fluid. A good level of amplification is obtained for the target sequence on the A1AT gene ( $\alpha$ ) but substantial cross-contamination is evident from the amplification level obtained for the last control segment  $C_1$ , following  $\alpha$  (containing no DNA template but the same concentration of primers and reagents contained in  $\alpha$ ) if compared to the signal obtained from the control segment  $C_1$ , which precedes  $\alpha$ . (B) The introduction of a plug of water between two consecutive samples reduces cross-contamination to an insignificant level.

aqueous plug was loaded with Bromophenol blue as a front sample.

Theoretically, an aqueous sample droplet in a continuous flow of an organic immiscible liquid in a hydrophobic channel will travel separated from the adjacent one, even at high velocities. Instead of disintegration into smaller droplets or leaving a thin film behind as in the case of wetted surfaces, the droplet detaches from the surface where a layer of the organic phase is created between the channel surface and the droplet. This is fundamentally interesting, since wall interaction with the analytes and the reagents is eliminated due to droplet levitation. An additional benefit of this situation is that the friction between the two phases leads to an intrasegmental movement within the droplet, resulting in effective mixing.<sup>39</sup> However, the practical results from our experiments showed a considerable cross-contamination. The reason for this behavior seems to be complex. Possible factors to be considered are adsorption of analyte or reagents, surface imperfections of the tube, the high temperature involved (close to the boiling point of water), and the length of the organic solvent plug versus the length of the sample plug. Model experiments with sample plugs of an aqueous solution of 2',7'-difluorofluorescein, where we studied the influence of temperature, flow speed,

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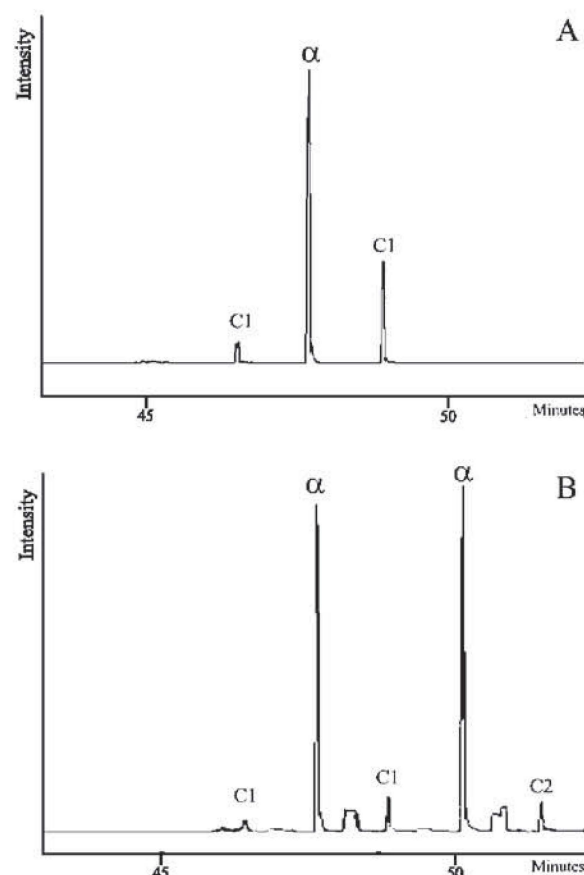


Figure 3. (A) PCR results performed with the same analytes and reagents as in Figure 2A but with perfluorodecalin as transport liquid. The sample carryover is still present although notably reduced (cf. Figure 2A). (B) When a water plug is introduced between samples, cross-contamination is reduced to an insignificant level.

and length of the organic plug, showed a tendency toward increased carryover when operating at increased flow rates and elevated temperature (95 °C), but the main factor affecting the carryover was the plug length of the organic liquid. Further observations revealed that minute droplets seem to detach from the aqueous sample. These droplets travel at a slightly lower speed than the aqueous sample and are gradually picked up by the next sample plug. The small peaks observed after the last  $C_1$  peak in Figure 2A may, therefore, be signals resulting from plug break up.

The results of the experiments thus indicate that one way to reduce carryover would be to increase the plug length of the organic liquid. However, this would also reduce the throughput capacity of the system. A better strategy was to insert water plugs (1  $\mu$ L) between the samples while maintaining the distance (30 cm) between the sample plugs; the carryover problem was largely eliminated. The water plugs have the function of collecting the minute trailing droplets from the preceding samples and diluting their content. The improved results are shown in Figure 2B. The difference between the signal strength for  $\alpha$  and for the last  $C_1$  plug is more than adequate for distinguishing between amplified and nonamplified DNA.

To further improve the performance of the system, we utilized perfluorodecalin (boiling point 142 °C) as carrier liquid. Perfluorocarbons are substantially more hydrophobic than hydrocarbons.



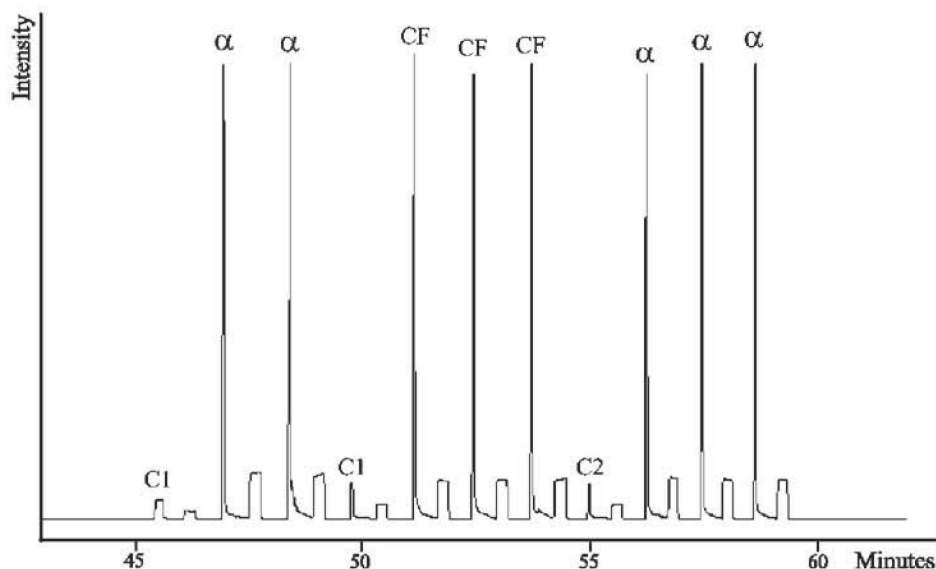


Figure 4. Example of a longer series of PCR amplification, using perfluorodecalin as transport liquid and a water plug between each sample: ( $\alpha$ ) amplified target sequence of the A1AT gene; (CF) amplified target sequence of the CFTR gene; ( $C_1$ ) Reference sample containing the same concentration of reagents contained in  $\alpha$  but no DNA template; ( $C_2$ ) reference sample containing the same concentration of reagents contained in  $\alpha$  but no DNA polymerase.

Thus, the interfacial surface tension between the aqueous sample and the carrier liquid will be increased, which should counteract a disintegration of the sample plugs. Additionally, the solubility of water in perfluorocarbons is extremely poor, and they show very poor affinity toward biomolecules.<sup>40</sup>

A significant improvement regarding carryover was indeed observed (Figure 3A), but the intensity of the signal obtained in the last  $C_1$  peak is still unacceptably high, although the small trailing signals, as observed in Figure 2A, are not detected anymore. In this context, it should be noted that the strength of the signals for  $\alpha$  and the last  $C_1$  peak does not reflect a volumetric proportion of fluid carryover. In fact, DNA amplification during the 30 cycles is not a linear multiplication, but rather a process with a logarithmic rolloff, due to reagent depletion, etc. It is therefore likely that the DNA traces, which are picked up by the following  $C_1$  plug, are amplified under more optimal conditions and will result in proportionally more template copies. It is clear that a water plug between consecutive samples is still required in order to suppress cross-contamination. The results are shown in Figure 3B. In this experiment, besides  $C_1$ , a second reference sample ( $C_2$ ) containing the DNA template but no DNA polymerase was introduced. Figure 4 shows an example of amplification of a sequence of samples, with the fluorocarbon as carrier liquid and water plugs between the samples, where a second amplification target (148 bp) from the CFTR gene was included and successfully amplified. It can be seen that the DNA amplification is quite reproducible between individual samples and that cross-contamination is reduced to a low level. The signals observed between the peaks are due to a background response of analyte in the water plugs. The plateaulike appearance of these signals is due to the extended dwell time, since these plugs are 3 times longer than the sample plugs.

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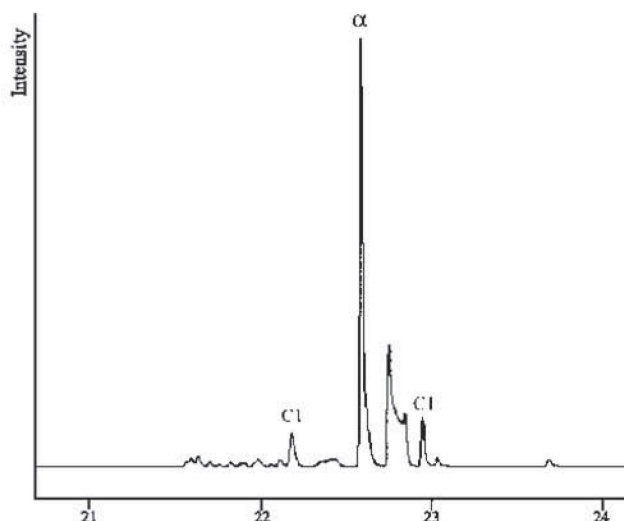


Figure 5. Segmented-flow PCR amplification, performed under the same experimental conditions as in Figure 3B, but with the flow speed doubled. Amplification of the target sequence on the A1AT gene ( $\alpha$ ) is still good, with a very low level of carryover.

The setup can be kept running continuously without any particular care, and the fluorocarbon can be periodically recycled from the collection vessel after separation from the aqueous phase. It is possible to run with considerably higher flow rates, thereby increasing the sample number capacity/time unit. Good results (see Figure 5) were still achieved in half the time frame of the earlier experiments. The time spacing between each sample was only 20 s. When the flow speed was further increased, the signal strength obtained after amplification started to decrease, while a larger signal of the intermediate water plug was obtained. Thus, carryover seems to increase. Also, the heat transfer through the Teflon tubing and a proper addition of the intercalating dye may become limiting factors.



It is clear that the physical design of the system could be further optimized. The use of miniaturized fluidics on a chip-based format is particularly attractive. It should be possible to significantly shorten annealing and denaturing times, as well as to reduce the number of amplification cycles.<sup>19</sup> However, such improvements are not generally applicable and have to be carefully evaluated for each individual application. In this context, it is interesting to note that the throughput capacity of the segmented-flow principle is not dependent on the tube length but only on the sample plug length, the distance between consecutive samples, and the flow speed.

To increase the total sample capacity, many flow channels could be operated in parallel. However, this requires another detector. Successful preliminary experiments with a detector based on a liquid core waveguide principle<sup>41,42</sup> have already been performed. Also, other parallel formats for the fluidics could be employed, e.g., stacked plastic disks of a CD-like shape with micromachined channels in a spiral form. Further, it would be of interest to collect the individual sample plugs for specific analysis. This will be reported in a forthcoming paper. Combined with a suitable automated system for handling and introduction of very small sample volumes, the continuous segmented-flow PCR concept should be a candidate for extremely high-throughput genetic analysis with minimum consumption of reagents and associated reduced cost.

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## CONCLUSIONS

The presented approach of continuous segmented-flow PCR has been shown to provide a basis for rapid and miniaturized DNA amplification. A particular feature of the technology is that the throughput capacity of the system is only dependent on the sample plug length, the distance between the samples, and the flow speed. Also, the simplicity of the setup is one of the attractive characteristics. A water plug between each sample segment was necessary to minimize carryover. The use of the fluorocarbon perfluorodecalin further improved the stability of the sample plugs. Using a single Teflon capillary, it was possible to amplify DNA samples in volume segments of 300 nL, at a rate of 20 s/sample. The use of the intercalating dye PicoGreen, added after the completion of the reaction, and detection by optical fiber-based LIF allowed an immediate on-line registration of amplification. Parallel operation of a large number of flow reactors should provide a system with a very high throughput capacity, which should have considerable potential for large-scale genetic analysis and diagnostics.

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