

Articles

Real-Time Nucleic Acid Sequence-Based Amplification in Nanoliter Volumes

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Real-time nucleic acid sequence-based amplification (NASBA) is an isothermal method specifically designed for amplification of RNA. Fluorescent molecular beacon probes enable real-time monitoring of the amplification process. Successful identification, utilizing the real-time NASBA technology, was performed on a microchip with oligonucleotides at a concentration of 1.0 and 0.1 μM , in 10- and 50-nL reaction chambers, respectively. The microchip was developed in a silicon–glass structure. An instrument providing thermal control and an optical detection system was built for amplification readout. Experimental results demonstrate distinct amplification processes. Miniaturized real-time NASBA in microchips makes high-throughput diagnostics of bacteria, viruses, and cancer markers possible, at reduced cost and without contamination.

Applying microsystem technology to the diversity of analytical problems has become an area of enormous interest, especially in connection with the development of microfluidic chips for clinical and forensic analysis.¹ One advantage of miniaturization is sub-microliter consumption of reagents and sample. In addition, improved heat- and mass-transfer rates may give faster reaction kinetics. Miniaturization enables integration of multiple analytical steps in the same device, thus reducing the risk of carryover contamination. Hand-held lab-on-a-chip devices for point-of-care diagnostics are being developed.

A commonly used technique in molecular biology, clinical research, and evolutionary studies is enzymatic amplification of nucleic acids. The first thermostable amplification procedure

published, polymerase chain reaction (PCR),² allowed amplification to a great number of copies of a specific region of a DNA chain in a very short time. Northrup et al.³ initially introduced PCR in silicon microstructures in 1993. Since then, numerous publications have appeared on simplification of PCR in microsystems using different approaches.^{3–15} Most of the reported PCR amplification methods use a combination of silicon and glass chips, with reaction chambers in the microliter range. Only a few reports describe PCR in nanoliter volumes or smaller. Experiments by Nagai et al.⁴ have demonstrated successful PCR amplification in reaction chambers for volumes down to 86 μL . Amplifications in reaction volumes of 160 and 280 nL have been reported by Hühmer and Landers and Lagally et al., respectively.^{5–8}

The main benefit of reducing sample volumes in PCR lies in enhanced thermal- and mass-transfer rates, which can significantly reduce the reaction time. Different approaches have been reported in order to obtain efficient heat transfer, such as conventional thermocyclers,⁹ integrated polysilicon thin-film heaters,³ Peltier elements,¹⁰ infrared radiation,⁵ waterbaths,¹¹ copper blocks,¹² and indium–tin oxide thin-film heaters.¹⁵

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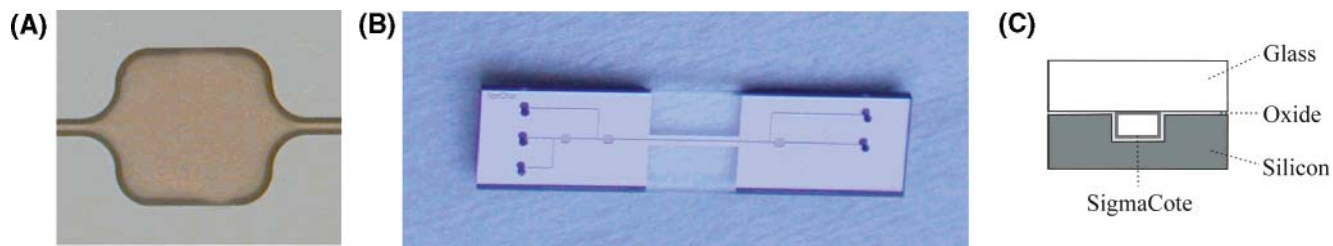


Figure 1. (A) Photograph of a 10-nL reaction chamber, $450 \times 450 \times 50 \mu\text{m}$. (B) The dimensions of the outer microchip are $5000 \times 20000 \mu\text{m}$. The two additional reaction chambers and channels were intended for loading of different reagents but were not used in these experiments. (C) Sketch of the cross-sectional area of the microchip.

The development of PCR in microsystems has led to the integration of complex procedures relevant for performing on-chip PCR. Microchips where several analytical steps were incorporated onto a single device have been reported, including the following: cell lysis, amplification, real-time detection, and electrophoretic separation of PCR products.^{4,5,8,13,14}

We have applied an alternative amplification method, termed nucleic acid sequence-based amplification (NASBA), to microchips. NASBA, initially introduced by Compton¹⁶ in 1991, is a sensitive, transcription-based amplification system specifically designed for detecting RNA. The technology relies on the simultaneous activity of three enzymes (avian myeloblastosis virus reverse transcriptase, RNase H, T7 RNA polymerase) under isothermal conditions (41°C), producing more than 10^9 copies in 90 min. The amplification method is particularly well suited for analyses of various kinds of RNA: genomic RNA, mRNA, rRNA, viroids, and ssDNA. In some NASBA systems, dsDNA may also be amplified, albeit very inefficiently, and only in the absence of the corresponding RNA target.¹⁷ Based on this, the NASBA reaction has an application range including viral diagnostics, gene expression, and cell viability.¹⁸

NASBA is isothermal and consequently no thermocycling is needed. This is an advantage since it simplifies both the microchip design and the instrument specifications. In NASBA, the amplification is dependent on three enzymes, each catalyzing a specific reaction. Optimal stoichiometric ratio of the enzymes involved is necessary for the reaction to proceed. Thus, the amplification reaction itself is more complex in the case of NASBA than in the case of PCR, which only utilizes one enzyme. In NASBA, molecular beacon probes^{19–21} hybridize to the target during the amplification, making possible real-time monitoring, which simplifies both the analytical procedure and the features of the microchip.

In this work, we report successful real-time amplification of oligonucleotides using NASBA technology in 10- and 50-nL silicon–glass reaction chambers. To our knowledge, this is the first time NASBA has been demonstrated in such a microsystem format.

MATERIALS AND METHODS

Microchip Fabrication. The microchips were processed by SINTEF. Chambers and channels were etched in the silicon wafers with a $\langle 100 \rangle$ crystal orientation using reactive ion etching. A 700-\AA oxide layer was grown, before the silicon wafers were bonded to $525\text{-}\mu\text{m}$ -thick Pyrex glass, forming channels and chambers. The channels have cross sections of $50 \times 50 \mu\text{m}$. The dimensions of the 10- and 50-nL reaction chambers were $450 \times 450 \times 50$ and $1000 \times 1000 \times 50 \mu\text{m}$, respectively. Conically shaped holes in the Pyrex wafer were made by powder blasting by Micronit. The diameters of these holes were $430 \mu\text{m}$ on the top surface and $150 \mu\text{m}$ on the bottom surface. To prevent adsorption of template and inhibition of the enzymes, the chips were coated with SigmaCote (Sigma Chemical Co., St. Louis, MO), according to the manufacturer's instructions. Figure 1 shows photographs of a 10-nL reaction chamber (A), the whole microchip with dimensions of $5000 \times 20\,000 \mu\text{m}$ (B), and an illustration of the cross-sectional area of the silicon–glass microchip (C). The 50-nL microchips had the same layout as the 10-nL microchips, but with larger reaction chambers. Altogether, less than 70 microchips were fabricated for both 10 and 50 nL.

Optical Detection System and Heat Regulation. An optical system for measuring fluorescence was made for excitation at 494 nm and detection at 525 nm. The instrument consisted of a sample stage and a hinged optical table, located directly above the stage. The stage was mounted on an optical bench and had micrometer screws for x , y , and z -alignment of the sample.

Figure 2 shows a diagram of the optical geometry of the instrument. A high-intensity blue light-emitting diode (LED) (Marl International Ltd.) excited the fluorophores from above at a 23° angle to the reaction chamber. The excitation light was filtered and focused onto the reaction chamber. Emitted fluorescent light was collected by two lenses (Melles Griot, Santa Clara, CA) perpendicular to the reaction chamber and guided through a prism (Melles Griot), a dichroic beam splitter (Chroma Technology Corp, Brattleboro, VT), a filter (Chroma Technology Corp.), and finally into the photomultiplier tube detector (Hamamatsu). The data collection and preparation of the detected signal was processed on a laptop computer using LabView 5.11 software (National Instruments, Austin, TX). A schematic overview of the experimental setup is shown in Figure 3A. Figure 3B shows a photograph of the actual detection unit.

The intensity of the fluorescent light (3.5 pW) is extremely low compared to the excitation light (1 mW). Typically a filter transmits $\sim 1/10\,000$ of unwanted light, which is insufficient to

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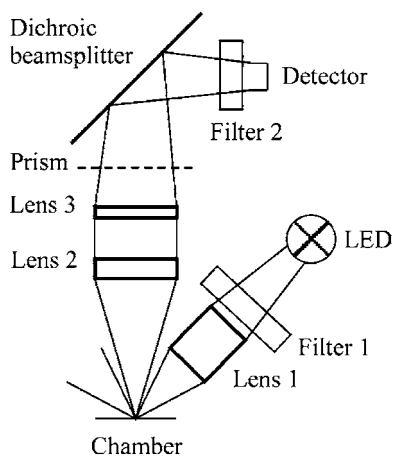


Figure 2. Sketch of the optical geometry. Blue light is emitted from the LED as shown in the diagram. Filter 1 is a bandwidth filter (465–500 nm). Lens 1 focuses the light onto the reaction chamber. The lens has a focal length of 10 mm and a diameter of 6 mm. Lens 2 (focal length, 17 mm; diameter, 14 mm) and lens 3 (focal length, 55 mm; diameter, 14 mm) collect and guide the fluorescent light from the fluorophores to a prism and dichroic beam splitter. The latter projects the light onto filter 2 (500–545 nm), which is mounted in front of the detector.

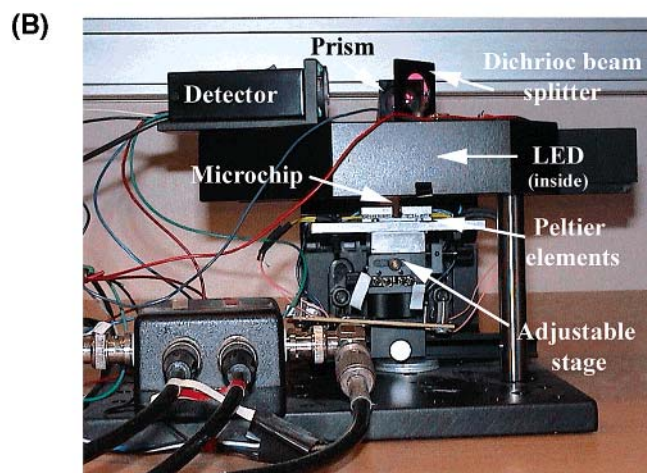
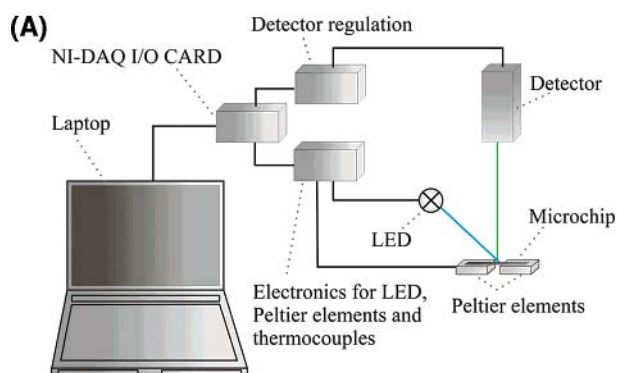


Figure 3. (A) Diagram of the experimental setup. (B) Photograph of the optical module.

separate the fluorescence from the LED light. Consequently, reflection or scattering of the excitation light into the direction of the optical path of the detector must be avoided. The 23° angle between the LED and the reaction chamber surface eliminates such reflections. To eliminate scattering, the surface in the

reaction chamber was made optically smooth, which means a surface roughness less than 1/10 of the wavelength of the light employed. The roughness in the reaction chambers in the silicon–glass chips was measured with a WYKO white light interferometer (Veeco Instruments Inc., Woodbury, NY) and found to be less than 40 nm and thus within the limits of optical smoothness.

To control the temperature of the chip, an aluminum chip holder was mounted on top of a Peltier element (Marlow Industries Inc., Dallas, TX). A thermocouple was integrated in the aluminum block with a feedback circuit to the Peltier element. The temperature system was controlled externally on a laptop computer with incorporated digital PID controllers (National Instruments) for regulation. The temperature precision of the system was within 41.0 ± 0.1 °C. A commercial Fluke temperature calibration apparatus (Fluke, Everett, WA) was used to calibrate the system with thermocouples and a platinum resistance sensor. Measurements were performed both on the aluminum block and on top of a dummy chip without glass. The Fluke temperature calibration unit measured absolute temperatures to within ± 0.1 °C. The overall temperature accuracy of the system was ± 0.3 °C, after calibration.

A limited number of disposable microchips were fabricated. Commercially available glass capillaries (Drummond Scientific Co, Broomall, PA) were used for temperature calibration, sample alignment, and testing of the data collection system. For these purposes, solutions containing active fluorophores in addition to the NASBA reaction mixture were applied to the glass capillaries. The glass capillaries had a capacity of 5 μ L with an inside diameter and outside diameter of 447 and 940 μ m, respectively. During measurements, only 2 mm of the capillary was illuminated; this corresponds to a detection volume of 300 nL.

Additionally, conventional 20- μ L NASBA reactions were performed in polypropylene tubes. The amplification was performed in a Biotek FL600 reader (MWG Biotech AG). The experiments were carried out in order to compare the experimental results from the microchips and the glass capillaries with conventional methods. The Biotek FL600 reader had a temperature variance of 41 ± 1 °C. Both the custom-made instrument with integrated thermal control and optical detection and the Biotek FL600 reader had an excitation wavelength at 494 nm and an emission wavelength at 525 nm.

Sample Material. A positive control for human papillomavirus (HPV) 16, from the HPV Proofer kit (NorChip AS, Klokkearstua, Norway) was used as sample material. In addition, an artificial 118-bp single-stranded DNA (ssDNA) 5'-GATTAGACATTTTCAGCATACGCATAATCGGCCGCTTCGCCTAGGCATATCCTT-TGCATGCTACTATATGGGACGATACGCCAAATGCCA-GTCAGATAGCACAGTAGCAGCGATTAA-3' (NorChip AS) was used to test NASBA in nanoliter volumes.

NASBA. The NASBA reaction was performed in microchips and glass capillaries with volumes of 10, 50, and 300 nL. For performance comparison, conventional amplification was carried out in 20- μ L polypropylene tubes.

Primers and molecular beacon probes for the HPV 16 were provided with the HPV Proofer kit (NorChip AS). Primers and probes for the 118-bp ssDNA were not included in the original kit. The following sequences were used in the amplification process of the ssDNA: primer 1 (5'-AATTCTAATACGACTCAC-

TATAGGGAGAAGGGCTGCTACTGTGCTATCTGA-3'), primer 2 (5'-GACATTTTCAGCATACGCATA-3'), and molecular beacon probe (5'-FAM-GCGGCATCCTTTGCATGCTACTATA GCCGC-dabsyl-3') (NorChip AS).

The reagents were mixed according to the manufacturer's instructions. It should be pointed out that manual mixing of reagents may lead to some relative shifts in the negative and positive baseline signals presented in the plots due to concentration variations of reagents. Depending on the application and target of interest, the reactants were optimized. For HPV 16, the final concentration of the molecular beacon was 0.42 μM , whereas for the ssDNA the concentration was 0.21 μM . As a negative control, water was added to the reaction mixture instead of target DNA. In addition to the regular kit reagents, yeast tRNA (Sigma Chemical Co.) was added to the reaction mixture to a final concentration of 4 $\mu\text{g}/\text{mL}$. To reduce the surface adsorption of enzymes and targets, tRNA was used as a dynamic coating. The surfaces of the silicon chips may inhibit the amplification reaction and were treated with surface agents to reduce nonspecific adsorption of the NASBA reagents. As previously described, both the silicon-glass chips and the glass capillaries were coated with SigmaCote to prevent adsorption.

Reagent solution (10 μL) from the kit and 5 μL of sample material (0.1 and 1.0 μM) were mixed and heated to 65 $^{\circ}\text{C}$ for 5 min. The mixture was subsequently cooled to 41 $^{\circ}\text{C}$, after which the enzymes were added and the resulting solution was kept at 41 $^{\circ}\text{C}$ for 5 min. A Hamilton glass syringe, with a disposable sequencing pipet tip attached to it, was used to apply the sample to a chip. The solution was drawn into the hydrophilic microchip by capillary forces. The inlet holes were subsequently covered with wax to avoid evaporation of the sample. The chip was incubated in the chip holder on top of the Peltier elements at 41 $^{\circ}\text{C}$. Approximately 10 min was needed to inject the reaction mixture into the reaction chambers and to align the microchip. The microchip was only used once due to a high risk of contamination if the microchips were to be used in subsequent experiment.

The same approach that was used for the microchips was utilized to coat, fill, and seal the disposable glass capillaries. The pipets were completely filled with reaction mixture and placed on the aluminum block on top of the Peltier elements underneath the optical detection system. The custom-built instrument detected only a 2-mm cross section of the glass capillary, corresponding to a reaction volume of 300 nL.

RESULTS AND DISCUSSION

The main objective of these experiments was to demonstrate the NASBA procedure in microchips with nanoliter reaction volumes. Due to the limited number of silicon-glass microchips, which passed the quality control, it was decided to test only one kind of sample in the 10- and 50-nL reaction chambers. Table 1 lists the experiments performed in microchips, glass capillaries, and polypropylene tubes. The results of the nanoliter-scale amplification reactions were compared to conventional NASBA performed in polypropylene tubes (20 μL).

The reactions presented in Figures 4–7 started after 10 min, due to the time consumed for addition of enzymes and injection of sample into the microchip and alignment in the instrument. Figures 4 and 5 demonstrate results for real-time NASBA per-

Table 1. Overview of the Figures Presented and the Experiments Performed

Figure	reaction chamber	chamber volume	sample material
4	glass capillary	300 nL	ssDNA
	polypropylene tube	20 μL	ssDNA
5	glass capillary	300 nL	HPV 16
	polypropylene tube	20 μL	HPV 16
6	microchip	50 nL	ssDNA
	polypropylene tube	20 μL	ssDNA
7	microchip	10 nL	HPV 16
	polypropylene tube	20 μL	HPV 16

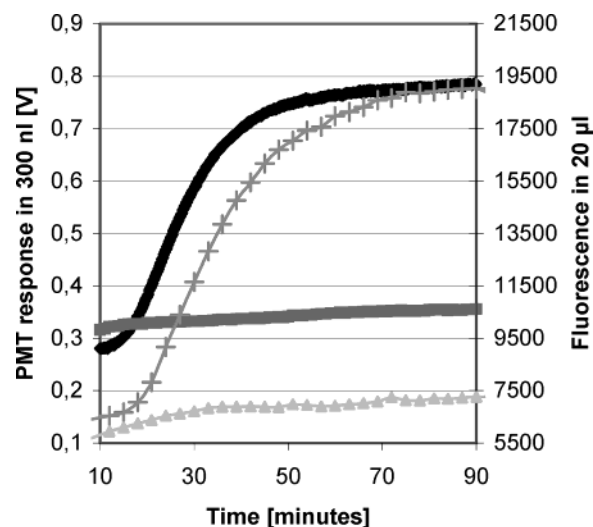


Figure 4. Real-time NASBA of ssDNA performed in glass capillaries and in conventional polypropylene tubes: \blacklozenge , 0.1 μM ssDNA in 300 nL; \blacksquare , negative control in 300 nL, + 0.1 μM ssDNA in 20 μL ; \blacktriangle , negative control in 20 μL .

formed in glass capillaries and in the conventional Biotek FL600 reader, using 0.1 μM ssDNA and 1 μM HPV 16 as sample material, respectively. The results using negative controls are presented in each case.

A comparison of the curves shown in Figures 4 and 5, for 300 and 20 μL , displays a high degree of conformity in performance. The graphs demonstrate the characteristic shape of a real-time amplified reaction, and there is a clear difference between the amplification and the negative control. The exponential phase for detection starts at the same time for both 300- and 20- μL volumes. However, there is a significant difference in the signal level for the HPV 16 compared to that of the ssDNA caused by concentration variations of the molecular beacons. Using ssDNA as target, the signal levels obtained for both glass capillaries measured in the custom-made instrument and in the conventional reader demonstrate a 3-fold increase from start to end point. The increase of the conventional amplification of HPV 16 is ~ 7 times the base signal, whereas in the glass capillaries the increase is 5 times (Figure 5). This is expected, as noise will become more significant as the reaction volume decreases and will dominate at low signal levels.

Figure 6 shows results from experiments using ssDNA in silicon-glass microchips with 50-nL reaction chambers, and in 20- μL polypropylene tubes using the conventional reader. The ssDNA concentration was 0.1 μM . For illustration purposes, the

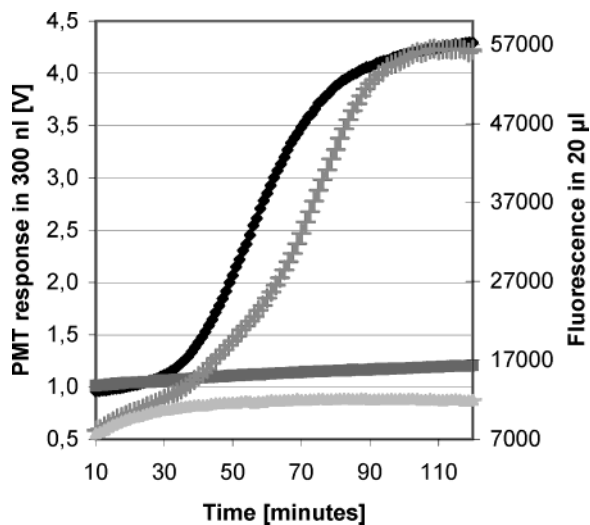


Figure 5. Real-time NASBA of HPV 16 oligonucleotides performed in glass capillaries and in conventional polypropylene tubes: \blacklozenge , 1.0 μM HPV 16 in 300 nL; \blacksquare , negative control in 300 nL, + 1.0 μM HPV 16 in 20 μL ; \blacktriangle , negative control in 20 μL .

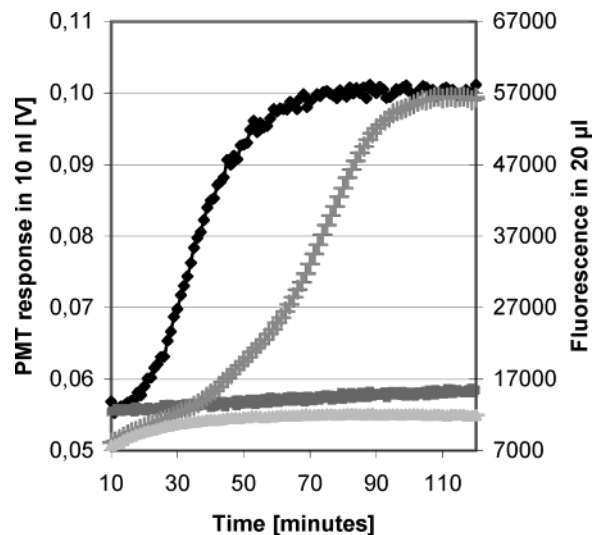


Figure 7. Real-time NASBA of HPV 16 oligonucleotides performed in a 10-nL reaction chamber and in conventional polypropylene tubes: \blacklozenge , 1.0 μM HPV 16 in 10 nL; \blacksquare , negative control in 10 nL, + 1.0 μM HPV 16 in 20 μL ; \blacktriangle , negative control in 20 μL .

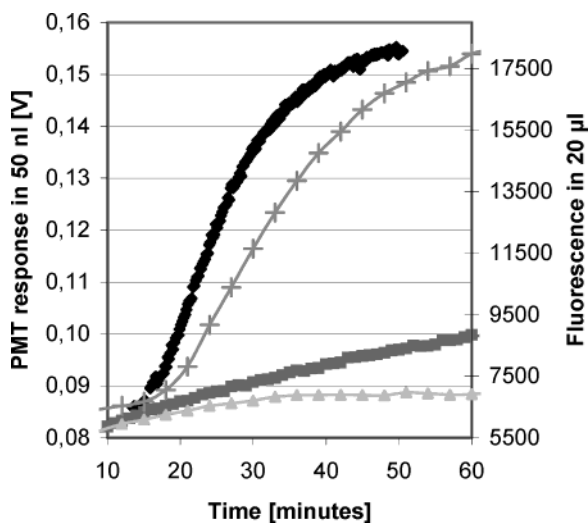


Figure 6. Real-time NASBA of ssDNA performed in a 50-nL reaction chamber and in conventional polypropylene tubes: \blacklozenge , 0.1 μM ssDNA in 50 nL; \blacksquare , negative control in 50 nL, + 0.1 μM ssDNA in 20 μL ; \blacktriangle , negative control in 20 μL .

negative control was adjusted by a factor of 0.45 in the figure. As shown in Figure 4, the conventional amplification signal increases 3 times from the starting level. In 50 nL, the signal increases by a factor of 2. The discrepancy between the negative control and the starting point of the amplified target was anticipated, as it was difficult to repeatedly place the microchip manually in exactly the same position every time. The error introduces a relative shift in the detector readout and does not affect the amplification process itself. The results show a distinct difference between the negative control and the amplification curve. The amplification curve displays the expected shape and time dependency.

Figure 7 shows the results of the amplification of HPV 16 performed in a silicon–glass microchip with a 10-nL reaction chamber and in the conventional polypropylene tubes. For the 10-nL reaction volume, the signal was amplified by a factor of 2 from 0.055 to 0.100 V. The fluorescence signal in the con-

ventional reaction volume was increased 7 times and is the same as presented in Figure 5. The 10-nL amplification curve has a different progress further to the left in the chart compared to the conventional curve. Several factors can result in the observed shape of the curve. Such factors could be due to higher target concentration¹⁷ and concentration variations, because the target of interest was acquired from different samples. In addition, the heat transfer in silicon is significantly faster than in glass or polypropylene and can result in reduced amplification times.

Another challenge related to microchip miniaturization is surface treatment. In microsystems, the surface-to-volume ratio is several orders of magnitude larger than in conventional systems. Preliminary experiments performed with no surface treatment of the silicon–glass structures gave negative results. Therefore, if the surface is not treated to prevent adsorption, the large surface area can disturb and even inhibit the whole process as in this case. Shoffner et al.²² emphasized the need of surface treatment to be able to perform PCR in silicon–glass structures. Enzymes are complex molecules consisting of hydrophilic, hydrophobic, and charged areas in a vital three-dimensional structure, and the surface properties of the reaction chamber can cause the enzymes to adsorb to the surface. The major subprocesses constituting the overall protein adsorption are changes in the state of hydration, redistribution of charged groups, and rearrangements in the protein structure. It is therefore important to obtain a surface with hydrophilic properties similar to that of the enzyme exterior.²³ The surface property in microsystems used for the NASBA reaction, which contains three enzymes, is therefore extremely important. Downscaling the reaction volume also effects the fluid dynamics of the system. The surface tension can be treated chemically to create either hydrophilic or hydrophobic behavior with specific liquids.²⁴

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CONCLUSION

The experimental results have shown it is possible to detect real-time NASBA amplification in 10-nL volumes by utilizing a custom-made microfabricated device and an optical detection system under process control. This is a reduction of the conventional 20- μ L reaction volumes by a factor of 2000. Furthermore, real-time NASBA was performed on two different target sequences at the nanoliter level. The performance of the NASBA reaction for the silicon-glass microchip was in agreement with the conventional method. But to obtain amplification in silicon-glass microchips, addition of small quantities of carrier molecules and surface treatment were required.

Miniaturization makes it possible to integrate processes such as amplification and detection within the same microchip. Integration of an additional function such as sample preparation will result in even shorter analysis time and in addition reduce the possibility

for contamination. The results from these experiments will be applied in future work toward an automated μ -TAS system for clinical diagnosis.

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