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Optimization of the performance of the polymerase chain reaction in silicon-based microstructures

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ABSTRACT

We have demonstrated the ability to perform real-time homogeneous, sequence specific detection of PCR products in silicon microstructures. Optimal design/processing result in equivalent performance (yield and specificity) for high surface-to-volume silicon structures as compared to larger volume reactions in polypropylene tubes. Amplifications in volumes as small as 0.5 μl and thermal cycling times reduced as much as 5-fold from that of conventional systems have been demonstrated for the microstructures.

INTRODUCTION

The ability to mass fabricate intricate, small-dimensioned structures using methods developed for the integrated circuit industry has significant implications for the manner in which many current analyses are performed (1,2). In this work we begin to examine the effects of miniaturization on aspects of genetic analysis, with a focus on PCR. The ability to perform small volume PCR in thermally conductive materials such as silicon offers the potential of rapid cycling, improved temperature uniformity, lower reagent costs and minimal sample requirements (3–8).

As one reduces the scale of the reaction, one increases the difficulty in handling materials and detecting products. In this work, we minimize these issues by employing an integrated thermal cycling and detection system in the microstructures (9–11). A homogenous fluorescent detection chemistry, 5′-nuclease assay (TaqMan™), allows the detection and quantitation of PCR products in real time. This technique employs a dye labeled probe technology and AmpliTaq Gold™ DNA polymerase. The probe is labeled with a fluorescent reporter dye and a fluorescent quencher dye. The proximity of the quencher to the reporter results in efficient quenching of fluorescence. The polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe is displaced from the target and the strand polymerization continues. The separation of the reporter and the quencher in solution results in an increase of fluorescence. This fluorescence is proportional to the amount of PCR product generated. The ability to directly quantitate amplification products using this chemistry in arrays of small volume silicon wells is the subject of this work.

MATERIALS AND METHODS

Silicon–glass microstructures

The silicon chips used to run PCR were designed to be 14 × 14 mm for compatibility with the CCD imaging system employed. Each contained an array of wells equipped with a 0.5 × 0.5 mm fill hole for manual loading of PCR sample and reagent, and a smaller vent hole to allow air to escape during filling. Optimizations were performed in 5 μl volume wells, arranged in a 2 × 4 array. A 0.70 mm thick silicon wafer was anisotropically etched to yield 0.5 mm deep wells. The fill and vent holes are then anisotropically etched from the opposite side of the wafer. A 4000Å layer of thermal oxide (SiO_x) is then grown on the etched surfaces of the wells. Finally, the chip is anodically bonded to a 0.5 mm thick borosilicate glass to provide an optical window into the top of the PCR well. After the addition of sample, the chips are sealed with an acrylic-based pressure-sensitive tape that is compatible with PCR chemistry (AR Care 7759, Adhesives Research, Glen Rock, PA). The sealed array is then clamped into the cycling/analysis system (Fig. 1) and the cycling protocol is started.

Materials

The β-actin template is the fully optimized standard for the ABI Prism™ Sequence Detection System (Model 7700). It uses human genomic DNA, representing a relatively complex PCR sample. The conditions examined for performance optimization were: magnesium concentration, primer concentration, probe concentration, AmpliTaq Gold (PE Applied Biosystems) versus AmpliTaq DNA polymerase, AmpliTaq Gold concentration, addition of a carrier protein (BSA, 007-BSA, New England Biolabs, Beverly, MA) and thermal cycling parameters (temperatures and times). Smaller volume silicon microstructures (down to 0.5 μl structures) were also examined.

The β-actin system (N808-0230, PE Applied Biosystems, Foster City, CA) provided in the kit specifies PCR in 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 3.5 mM $MgCl_2$, 400 mM dUTP, 200 mM each dATP, dCTP, dGTP, 300 nM each primer, 200 nM probe, 0.01 U/μl uracil-*N*-glycosylase and 0.025 U/μl AmpliTaq

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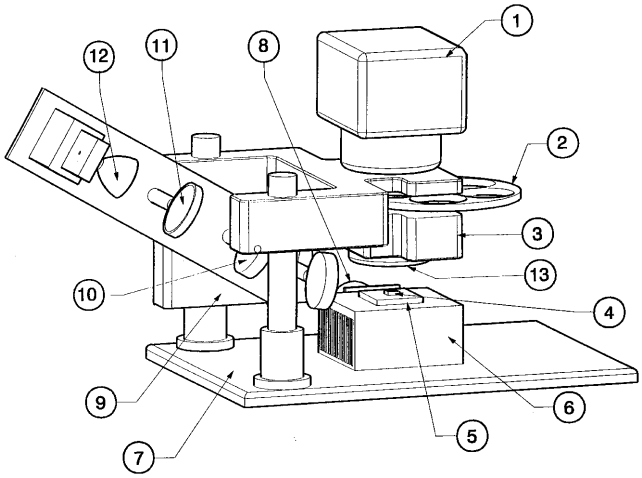

Figure 1. Schematic of breadboard instrumentation for real-time fluorescence detection of PCR products in silicon microstructures. 1, CCD camera; 2, emission filter wheel; 3, optics support; 4, silicon microstructure; 5 and 6, peltier and heat sink; 7, system base; 8, drive stage for microstructure positioning; 9 and 10, focusing lens; 11, excitation filter; 12, tungsten light source; 13, collection optics.

Gold DNA polymerase. The sequence for the β-actin forward primer was 5′-TCA CCC ACA CTG TGC CCC ATC TAC GA-3′. The sequence for the β-actin reverse primer was 5′-CAG CGG AAC CGC TCA TTG CCA ATG G-3′. Together these two primers define a 297 bp PCR product. For homogeneous detection using the TaqMan™ system, a fluorogenic probe was also included. The sequence of the β-actin fluorogenic probe was 5′-FAM-ATG CCC-X(TAMRA) CCC CCA TGC CAT CTC TGC TGC GT-PO_4-3′.

Detection of PCR products

Initial assessment of PCR performance was determined using capillary electrophoresis (Model 270A/HT, PE Applied Biosystems, Foster City, CA). The PCR product was diluted with distilled water (1:20) and electrokinetically injected into the capillary (–5 kV, 60 s). Separations were performed using a polymer media (401608, PE Applied Biosystems) and quantitated by UV detector at 260 nm. Size was determined by comparison to a DNA size ladder (401607, PE Applied Biosystems). PCR in polypropylene tubes was performed on the Models 2400 and 9600 (PE Applied Biosystems). The Model 7700 Sequence Detection System (PE Applied Biosystems) was used for the direct quantitation of signal from the homogeneous TaqMan™ probe in the tube controls. In this type of detection system the exponential phase is over before any product is detected (12).

The prototype instrument for product detection from silicon devices (Fig. 1; 13) consists of a tungsten bulb for illumination and a CCD camera and a four-color filter wheel for detection. During the thermal cycling, the progress of the PCR is monitored by taking images of the device at several wavelengths in order to monitor the increase of the reporter fluorescence (FAM) following PCR. The device is flood illuminated by the inexpensive, incandescent tungsten light source which is capable of achieving an irradiance of 5 mW/cm^2. The fluorescence is imaged 1:1 onto a thermo-electrically cooled instrumentation-grade CCD through one of the four interference filters mounted in a filter wheel (5 s integration). Both temperature control and fluorescence measurements are controlled by a LabView-based software package. The instrument is capable of binning signals from each well of the device. The signal results from changes in the fluorescence emission intensity of the reporter dye following cleavage of the probe. Signal normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the passive reference for a given well. The reporter signal intensity is measured at 518 nm, the passive reference is measured at 602 nm, and the excitation reference wavelength is 488 nm.

RESULTS AND DISCUSSION

General performance

PCR performance comparable to that obtained in standard-tube formats is attained in silicon devices coated with 4000 of thermal oxide. Typical yield for a chip is 75–125% to that of tube chemistries with a variability of <20% within a single 8-well chip. Similar yields for the PCR product with no significant non-specific products are observed as determined by capillary electrophoresis.

Methods for the β-actin TaqMan™ kit were evaluated and optimized at a number of volumes. The volumes evaluated were 0.5, 1.0, 2.0, 5.0 and 9.0 µl. No significant difference in yield (by capillary electrophoresis) or fluorescence level was observed over this volume range. Figure 2a shows real-time images of 5 µl silicon wells at various DNA concentrations; Figure 2b and c shows subsequent image and data analysis.

PCR optimization

Table 1 summarizes the parameters used to optimize PCR for the β-actin system. The effects of varying the parameter concentrations were evaluated by comparing the magnitude of the normalization ratio and the threshold cycles for defined template concentrations. Figure 3 graphically illustrates some of the effects; additional discussion on key issues is given below.

Table 1. PCR optimization in silicon devices

Variable	Range	Optimized concentrations in	
		Tube	Si device
Mg	2.5–5.0 mM	3.5	4.0
Primer	100–600 nM	300	300
Probe	50–250 nM	200	200
BSA	0–0.2% w/v		0.05
AmpliTaq™ Gold	0.025–0.25 U/µl	0.025	0.10

Magnesium. Amplification was observed in the 7700 system in all but the 2.5 mM concentrations; the prototype showed no amplification at the 2.5 or 3.0 mM concentrations. The 3.5 mM magnesium concentration on both the 7700 and the prototype showed considerable variability in the levels of fluorescence.

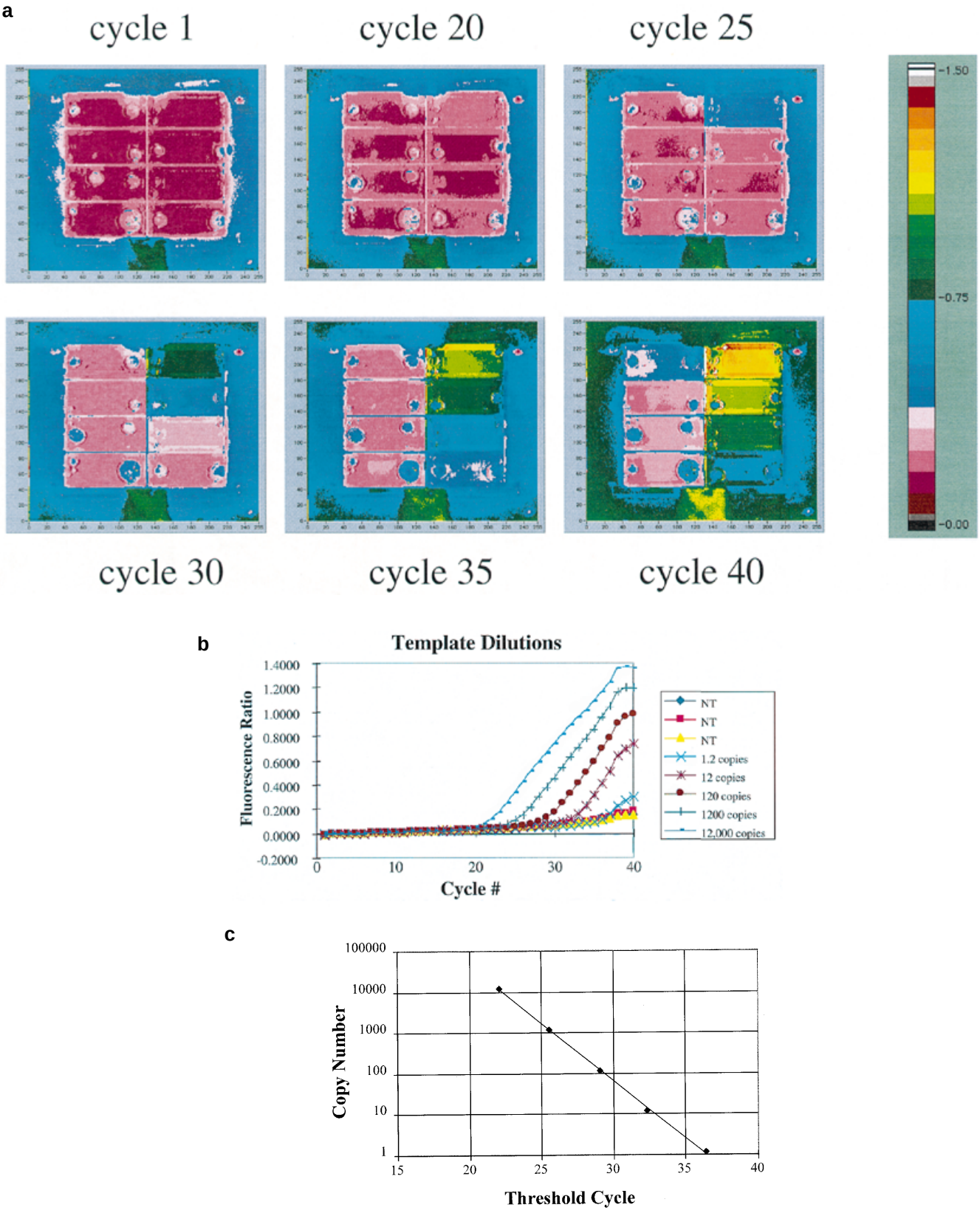

Figure 2. (**a**) Template dilution series: chip images at different cycle numbers. Loading from top to bottom: Left = 1.2 copies, NT, NT, NT; Right = 12 000, 1200, 120, 12 copies. Bar graph equals fluorescence signal ratio (FAM/ROX). (**b**) Baselined fluorescence ratio as a function of cycle number for a known dilution series using data generated in (a). (**c**) Efficiency of amplification: copy number as a function of threshold cycle (12). Best fit of all data, efficiency = 91%; excluding lowest copy number, efficiency = 95%. Efficiency = $10^{(\text{slope of best fit line})} - 1$.

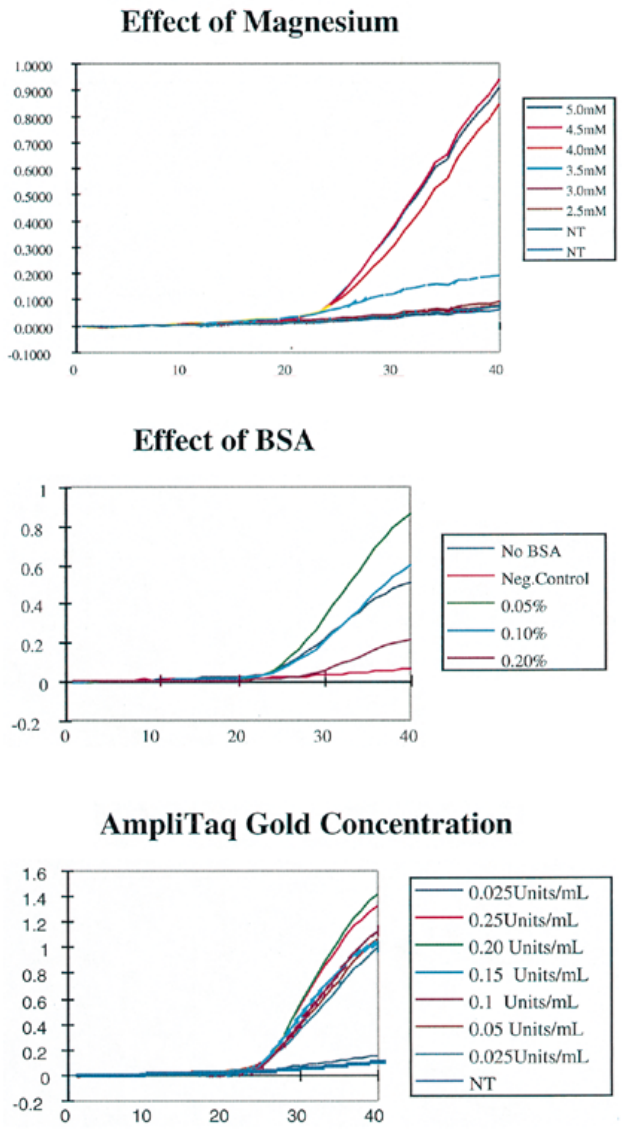

Figure 3. Optimization of β-actin. Baselined fluorescence ratio versus cycle number for specified parameter. X-axis, cycle number; y-axis, fluorescence ratio.

Therefore, a concentration of 4.0 mM was used for all further testing (Fig. 3, top).

Carrier protein. High concentrations of BSA (>0.15%) cause lower yields than the control (zero BSA) and can shut down the reaction at very high concentrations. At optimum conditions (0.05%) the BSA can increase the yield by nearly 2-fold (Fig. 3, middle).

AmpliTaq Gold™ versus AmpliTaq™ DNA polymerase. A comparison of AmpliTaq Gold™ versus AmpliTaq™ polymerase was also investigated (data not shown). The AmpliTaq Gold™ DNA polymerase generated an increase in fluorescent yield of ~35% compared to the AmpliTaq DNA polymerase. AmpliTaq Gold™ also improved the precision from a CV of 14 to CV 6% for the same data set.

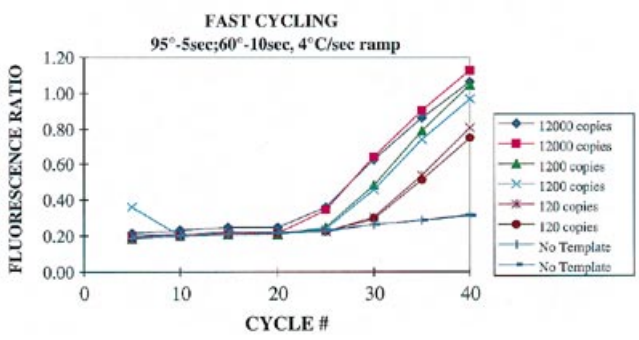

Figure 4. Baselined fluorescence ratio for optimized β-actin: effects of rapid cycling parameters.

AmpliTaq Gold™ DNA polymerase optimization. In order to obtain consistent PCR amplification in the silicon devices run on the breadboard system it was necessary to raise the AmpliTaq Gold™ DNA polymerase concentration from the recommended concentration of 0.025 U/µl used in the Model 7700 system with polypropylene tubes. There was amplification at all the different concentrations of enzyme on the 7700 for the β-actin template; however, on the prototype there was occasionally no amplification at the lower concentration of 0.025 U/µl. Therefore, a concentration of 0.1 U/µl was used for any further testing (Fig. 3, bottom).

Sensitivity: template dilutions

Dilutions of the target were investigated to assess system sensitivity. The concentration of AmpliTaq Gold™ used for these evaluations was 0.1 U/µl. A range from 12 000 to zero copies (no template, NT) of genomic DNA were assayed for β-actin. Results were comparable between the 7700 and the breadboard system. The β-actin assay clearly showed amplification down to the 1:1000 dilution (12 copies of template per reaction) and some signal at the single copy level (Fig. 2a and b). Bubbles can be identified in wells 1 and 2 of the cycle images but do not affect the results which are based on the ratio of signal at multiple wavelengths (4).

Thermal cycling conditions: rapid cycling

Optimized thermal cycling parameters for the β-actin assay are similar to those employed on the 7700 in a tube format. The optimal thermal cycling conditions are as follows:

Precycle: 50°C for 2 min, 95°C for 10 min.
Cycling: 40 cycles of 95°C for 15 s, 60°C for 60 s.
Hold: 72°C forever.

In attempts to further exploit the scale and thermal properties of the silicon structures, attempts were made to further reduce these cycle times. Figure 4 shows the analysis for an 8 well, 5 µl chip in which the temperature ramp rates are increased 4-fold to 4°C/s and the cycle times are reduced. The cycling parameters for the faster rates are 95°C for 5 s, 60°C for 10 s. This results in an overall cycle time of 32.5 s in the chip format versus 2 min, 35 s in tubes (a 5-fold reduction). Analysis of chip product yield by CE revealed only minor differences in PCR product yield between

normal and fast cycle times (peak areas of 13 000 versus 12 000). Occasionally a higher fluorescence reading at the initial cycle is seen, the cause of which is unknown.

CONCLUSIONS

The ability to perform and monitor arrays of PCR reactions in silicon devices has been shown to be feasible to submicroliter volumes with no loss in yield or specificity when compared with conventional protocols. Reactions in silicon substrates required addition of small quantities of carrier protein and/or higher enzyme concentration to obtain equivalent performance to tube chemistries. The addition of carrier protein is probably required to limit binding of enzyme to the surface walls.

PCR with real-time detection in microstructures is one key element in addressing throughput (more, faster reactions) and cost (lower materials needs) issues in genetic testing. Further work in this area will focus on improved consistency of the PCR reaction in batch production (process optimization, cleaning/handling procedures). In going forward attention needs to be focused on sample preparation, low volume fluid handling, and post-PCR analyses (14–18).

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