The role of DNA diffusion in solid phase polymerase chain reaction with gel-immobilized primers in planar and capillary microarray format

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The solid phase polymerase chain reaction (PCR) on a gel-based microarray system was studied under various durations of individual stages of the PCR cycle and spatial restriction of the reaction volume. Combining the experimental study with numerical modeling, we demonstrated that the diffusion of the PCR product in and out of a microarray element during the annealing and melting stages, respectively, is the main factor responsible for distinctive features of the studied type of PCR. The restriction of reaction volume leads to faster PCR signal growth. Particularly, the capillary array, whereby gel-based microarray elements are located on a glass bar inserted into capillary chamber, was found to be a suitable format for the development of the platform. © 2009 American Institute of Physics.

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I. INTRODUCTION

Since the invention of polymerase chain reaction (PCR) more than 2 decades ago,¹⁻³ it has been widely used in various applications of life sciences and its mechanism is thoroughly studied and described. However, there were no many studies on solid phase (heterophase) PCR (SP-PCR), where primers (or part of them) could be extended while immobilized on a solid support.⁴

In the most orthodox scheme of SP-PCR (also known as bridge PCR), both forward and reverse primers are immobilized on a support and no primers are present in the liquid phase.^{5,6} This scheme excludes any possibility of primer dimer artifact (which is particularly important in case of multiplex PCR) and is suitable for polymerase colony formation. Currently, the bridge PCR scheme is used in Solexa DNA sequencing technology. Compared to traditional PCR, the bridge PCR has extremely low yield. Theoretical study⁷ indicates that it cannot even be characterized by an exponential growth. This places serious limitations on practical use of this type of SP-PCR.

The situation could be improved by an addition of primers into a liquid phase. The SP-PCR with solid phase forward and liquid phase reverse (or vice versa) primers (Fig. 1) was utilized in a microtiter plate format.⁸⁻¹⁰ The combination of state-of-the-art picoliter-scale plate with the Sepharose bead substrate resulted in unprecedented parallelism of 300 000 independent SP-PCR amplification of randomized single DNA molecules.¹¹ This achievement was subsequently used in a novel technology of megascale DNA sequencing.¹²

An independent cohort of researchers has been working on implementation of SP-PCR to amplification of specific targets on microarray. The suitability of microarray-based SP-PCR for detection of mutations and polymorphisms,¹³⁻¹⁶ detection of toxin genes and microorganisms,^{13,17-20} eukaryotic expression profiling,²⁰ methylation analysis,²¹ and generation

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FIG. 1. The scheme of SP-PCR (for i th cycle). At the melting stage free and immobilized primers are totally separated from the extended forward primers [denoted as $g_i(x)$ in numerical modeling] and free PCR products [denoted as $f_i(x)$ in numerical modeling], respectively. At the annealing stage free reverse primers and free PCR products are annealed to their immobilized counterparts. At the elongation stage all the annealed primers are extended to form full length double stranded products detectable with SYBR Green (the latter is not shown).

of extremely long microarray probes²² has been successfully demonstrated. Some of these works utilized the traditional immobilization of solid phase primers on a functionalized glass surface, while the others used a polyacrylamide gel as a support.^{13,14,18–20} The latter approach was particularly successful as the immobilization in a gel separates a primer from solid support²³ facilitating the PCR.¹⁰ Despite that the SP-PCR is believed to have severe limitations compared to traditional PCR, as little as ten copies of microbial DNA were successfully detected and quantified in gel-based SP-PCR assay.¹⁹ Besides, the PCR curves could be measured on a gel-based microarray in real time. It was shown that similar to traditional liquid phase PCR, the SP-PCR with at least one primer in liquid phase could be characterized by exponential curve.^{19,20} Some important distinctive features were also found. In particular, PCR efficiency was found to depend on the size of the microarray elements (gel pads).²⁰ In these pioneering experiments the traditional aspects of PCR, such as standard curves and effect of buffer components, were also studied.

In contrast to those earlier studies, this work is focused on physical aspects, which distinguish SP-PCR and traditional liquid phase PCR, aiming the deeper understanding of solid phase reaction. Particularly, we considered the effect of microarray geometry and the durations of individual stages of PCR cycle. Based on these results, we supposed that diffusion of PCR product in and out of the “active zone” of reaction (i.e., the area of solid phase primer localization) is the key factor of this considered form of SP-PCR. To validate this hypothesis we introduced a novel design of microarray where the primer-containing spots of gel are located on a $0.3 \times 0.1 \text{ mm}^2$ glass bar inserted into a polypropylene capillary. This design restricted the diffusion of the PCR product out of the active zone of reaction and resulted in faster growth of PCR signal, in accordance with our hypothesis. To validate the hypothesis further we built a mathematical model of SP-PCR on a planar and capillary array. In combination with experimental data, the numerical modeling results confirm the hypothesis about the role of diffusion in of SP-PCR, thus providing a deeper understanding of this reaction.

II. MATERIALS AND METHODS

A. Primers

Primers were designed using Hytherm²⁴ web tool (Ref. 25) and DNA sequences available from public databases. Primers were synthesized on an ABI-394 DNA/RNA synthesizer (Applied

Biosystems, USA) by standard phosphoramidite method and purified by reverse phase high performance liquid chromatography on C18-Nucleosil ($5\ \mu\text{m}$, $4.6 \times 250\ \text{mm}^2$) columns (Sigma, USA). The reverse primer consisted of two parts: the 5'-T7 promoter part (introduced for better thermodynamic stability) and 3'-poly-T part, GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG (T)₂₅. This primer was used for both reverse transcription (RT) and PCR.

To immobilize primers inside gel pads, an amino group was introduced during synthesis using 5'-Amino-Modifier C6 (Glen Research, USA). The forward primers Actb and Hprt were designed to contain specific oligonucleotide sequences of human β -actin and hypoxanthine phosphoribosyltransferase genes, respectively, free from any essential internal hairpin structures: Actb-NH₂-TAT TTT GAA TGA TGA GCC TTC GTG and Hprt-NH₂-TAA GAA GTT TTG TTC TGT CCT GG.

B. Preparation of cDNA

Total RNA was isolated from K562 myelogenous leukemia cells with RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The reaction mixture for RT of total RNA contained $1 \times$ StrataScript buffer (Stratagene, USA), 0.2 mM each dATP, dCTP, and dGTP, 0.6 mM dUTP, 2 μM reverse primer, ten units StrataScript[®] Reverse Transcriptase, 20 units RNasin[®], and 10 μl of total RNA preparation per 10 μl reaction. The RT was performed at 42 °C for 90 min with final incubation at 95 °C for 10 min. One μl of 20-fold diluted cDNA preparation was used for further on-chip PCR.

C. Microarrays

Planar oligonucleotide microarrays were fabricated by spotting gel polymerization solutions containing different forward primers onto glass slide (Corning 2947 Micro Slides, Corning, USA) with subsequent photopolymerization.^{19,20} The fabricated microarrays consisted of spatially separated gel pads with 0.6 mm diameter.

The capillary microarrays comprised of two parts: the polypropylene capillary and the glass bar with gel pads bearing the immobilized primers. The Corning glass slide was sliced into 3 mm wide glass stripes and the latter were drawn under the flame of gas burner. The obtained $0.3 \times 0.1\ \text{mm}^2$ glass bar was immersed into Bind Silane solution (Amersham Bioscience, USA) for 10 min, then briefly rinsed with ethanol and water and air dried. The obtained glass bar was spotted with gel polymerization solution and treated as a planar microarray. The fabricated one-dimensional (1D) array consisted of spatially separated $0.6 \times 0.3\ \text{mm}^2$ gel pads. Polypropylene capillaries were drawn from 1 ml insulin syringes (Becton Dickinson, USA) to the diameter sufficient for tight insertion of glass bar.

D. PCR on microarrays

The PCR was performed inside a microarray chamber formed by sealing the microarray slide with a cover glass slide by a Frame-Seal (Bio-Rad, USA). The reaction mixture consisted of 70 mM tris-HCl (pH 8.6), 16.6 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.2 mM each dATP, dCTP, and dGTP, 0.6 mM dUTP (Sileks, Russian Federation), 0.2 μM reverse primer, 0.01% acetylated BSA (Fermentas, Lithuania), 0.5 unit DNA Polymerase (Sileks, Russian Federation), 0.6 μL of template cDNA, and $1 \times$ SYBR[®] green I (Molecular Probes, USA) per 25 μl reaction. After initial incubation at 94 °C for 2 min, 45 cycles (by default, 20 s at 94 °C, 45 s at 55 °C, and 43 s at 72 °C) were performed.

E. Data acquisition and image processing

For real-time measurements of SYBR[®] green I fluorescence, the experimental setup that was described earlier consisted of fluorescent microscope equipped with thermostable, a charge coupled device camera, a temperature controller,^{13,19,20} and specialized software IMAGEEXPRESS[®] (Biochip-EIMB, Russia). Images were acquired every cycle at the end of elongation step. For the subse-

quent analysis of PCR, the average intensities of all 3×3 pixel areas were plotted against the cycle number and normalized by the intensity of excitation light. The resultant data (F) were normalized further according to the following formula: $(F - F_{\min}) / (F_{\max} - F_{\min})$, where $F_{\min}$ and $F_{\max}$ are the minimal and maximal values of F , respectively, across the PCR curve.

III. RESULTS

A. PCR signal through the array element

The scheme of reaction studied in this work is outlined in Fig. 1. The sequence-specific forward primers are immobilized in the gel pads (microarray elements) while common reverse primer is present in the liquid phase only. At the melting stage, PCR primers and products are totally separated. At the annealing stage, free PCR products anneal to immobilized forward primers while the reverse primers anneal to those forward primers that were extended in previous cycles of PCR. At the elongation stage, all the primers annealed earlier with their counterparts are extended by polymerase.

For detailed study of the processes occurring in array element in the course of PCR, the images acquired at every cycle were stored and subsequently analyzed. It was found that the signal initially starts to grow at the center of array element. This means that the signal profile across the element of array is different at exponential phase of PCR and at plateau phase [Fig. 2(a)]. At the exponential phase the profile is sharp and narrow while it gets broader at plateau phase. As a consequence, PCR curves for the central part of array element and its distal part are different: The PCR curve for the central part has the lower threshold cycle value (C_T) [Fig. 2(b)]. This observation could not be attributed to nonuniform distribution of immobilized primers as it was earlier demonstrated that immobilized oligonucleotides are distributed uniformly in a gel pad.²⁶ Further steps undertaken to understand this phenomenon indicates that it is an intrinsic property of SP-PCR rather than an artifact of nonuniform primer immobilization.

At the exponential phase of PCR the signal was found to increase by factor of 1.59 ± 0.04 per cycle (average of four independent experiments) for both distal and central parts of an array element [corresponding to areas inside and outside of $1/2$ gel pad radius, respectively; Fig. 2(c)], i.e., the efficiency of SP-PCR was constant across array element. However, it was surprisingly low in comparison with conventional liquid phase PCR.

B. The effect of melting, annealing, and elongation stages durations

Due to the observed difference between threshold cycle values for central and distal parts of array element, the following data were obtained for the center of array element only, as this point corresponds to the minimal C_T and the fastest growth of PCR signal. The twofold decrease in annealing time (from 45 to 22 s with 43 s elongation time) resulted in the increase in the C_T value by 4 cycles (from 27 to 31). However, the similar change in elongation time (from 43 s to 18 s with 45 s annealing time) led only to insignificant (within 1 cycle) increase in the C_T value. The increase in melting time from 20 to 40 s virtually inhibited the PCR (i.e., no essential signal growth was detected in course of reaction). The control liquid phase PCRs with both forward and reverse primers for *Actb* in solution performed on the slide with no immobilized oligonucleotides did not show any significant change in threshold cycle for 20 and 40 s melting times (C_T difference was within 1 cycle). The decrease in melting time from 20 to 7 s resulted in negligible decrease in threshold cycle (by 1 cycle). However, the data obtained with 7 s could be poorly reliable due to the thermal inertia of the system and increased possibility of incomplete strand separation of PCR products (as discussed in Sec. IV). Based on these findings, 20 s was chosen as a basic melting time for further experiments, as it provides a sufficient rate of signal increase and reliable data interpretation.

C. PCR dependence on array geometry

As it was reported earlier,²⁰ the threshold cycle value of SP-PCR is strongly dependent on the physical size of microarray elements. In particular, the C_T values for *Actb* were 26, 30, and 39

FIG. 2. General characteristics of SP-PCR. (a) Fluorescent signal across the array element at exponential and plateau phases of PCR. (b) The PCR curves for the central and distal parts of array element. (c) The correlation of PCR signals in a cross section of array element for two consecutive cycles at exponential phase. The signal changes proportionally for central and distal parts of array element (marked as opened squares and closed diamonds, respectively).

FIG. 3. The design of capillary array.

cycles with 600, 450, and 250 μm diameters of gel pad, respectively. It was hypothesized that the effect is due to the diffusion of PCR product out of gel pad during the melting stage. To test this hypothesis we designed a novel type of microarray, where the elements were located on a narrow (0.3 mm width) glass bar inserted into polypropylene capillary (Fig. 3). Such a change in the array geometry should restrict the diffusion of the PCR product during the melting stage in the lateral direction and produce faster signal growth. For PCR performed with the two types of arrays in identical reaction conditions, the obtained C_T values were 23 and 30 for capillary and planar arrays, respectively. Thus the results are in agreement with the suggested hypothesis.

D. Threshold cycle value for different positions inside the array element

As the threshold cycle value was found to be different for different positions inside the microarray element [Fig. 2(b)], we calculated ΔC_T relative to the center of the microarray element and plotted it against the position in cross section [Fig. 4(a)]. Both capillary and planar arrays revealed a quasiparabolic dependence with ΔC_T increasing from the center to the edges of the element. However, for the capillary array the increase was weaker than for the planar one, suggesting that the effect might be related to the restricted diffusion of the PCR product in a capillary. To verify this possibility we performed PCR on the planar array with the addition of Actb forward primer (identical to the immobilized one) in the liquid phase. In this scheme of experiment, the PCR product was synthesized uniformly across the array element and its diffusion could no longer affect the reaction. In order to minimize SYBR green I fluorescence in the solution surrounding the microarray element, the forward primer was taken in concentration of 20% relative to the reverse one. In this experiment ΔC_T was virtually independent of the position inside the microarray element [Fig. 4(a)].

The analysis of experiments with 22 and 45 s annealing times revealed that the shorter annealing time led to the stronger variation of ΔC_T value across the array element. Although the difference was not as strong as between capillary and planar arrays, it was nevertheless reproducible for both Actb [Fig. 4(b)] and Hprt [Fig. 4(c)] genes.

FIG. 4. C_T variation across the microarray element. (a) Forward primer is Actb. Closed diamonds: planar array with immobilized forward primer and reverse primer in liquid phase. Opened squares: capillary array with immobilized forward primer and reverse primer in liquid phase. Bold line: planar array with reverse and forward primers in liquid phase (the ratio of primers concentrations in liquid phase is 5:1). (b) Forward primer is Actb, no reverse primer in liquid phase. Closed diamonds: 22 s annealing; opened squares: 45 s annealing. (c) Forward primer is Hprt, no reverse primer in liquid phase. Closed diamonds: 22 s annealing; opened squares: 45 s annealing.

E. Numerical model: Accounting for the melting stage only

We started numerical modeling with the simplest model, which accounted for the diffusion of the PCR product at the melting stage only. This model disregarded details of PCR product hybridization to immobilized primer and its diffusion at the annealing stage but explained the most of observed phenomena.

For the capillary array 1D model was constructed, where $f_i(x)$ and $g_i(x)$ denoted the amounts of the free PCR product and extended solid phase forward primer, respectively (Fig. 1), at the position x at i th cycle of PCR.

The initial conditions were

$$f_1(x) \equiv 1,$$

$$g_1(x) \equiv 0.$$

At the i th cycle the free PCR product that accumulated during the previous cycles diffuses at the melting stage, while after the elongation stage it increases by the amount of the “new” product synthesized on the extended solid state primers $g_{i-1}(x)$,

$$f_i(x) = D_m^M f_{i-1}(x) + g_{i-1}(x),$$

where D_m is an operator of convolution with fundamental solution of diffusion equation, $D_m f(x) = c \cdot \exp(-(x^2/r_m^2)) * f(x)$; r_m is a diffusion distance per 1 s, c is the normalization constant, and M is the duration of melting stage in seconds. The amount of extended solid phase primer at the i th cycle $g_i(x)$ is increased by the amount of solid phase primers extended on the templates of free PCR products annealed to the microarray element,

$$g_i(x) = g_{i-1}(x) + \theta(x) D_m^M f_{i-1}(x),$$

where $\theta(x)$ equals 1 if the position x belongs to microarray element and equals 0 otherwise.

This model describes only the exponential phase of PCR and implies that amplification capability of microarray element (or the amount of immobilized primer) is unlimited. For the planar array the f_i , g_i , and θ are the functions of two variables [$f_i(x, y)$, $g_i(x, y)$, and $\theta(x, y)$, respectively], and D_m is the operator of two-dimensional (2D) convolution.

The model demonstrates that after several iterations $g_i(x, y)$ assumes the stationary shape [Fig. 5(a)] with maximum in the center, similar to the experimentally obtained profile [Fig. 2(a), exponential phase]. Remarkably, this result was obtained in presumption of uniform distribution of primer [$\theta(x, y) = 1$] inside the array element.

Then $g_i(x, y)$ grows in every subsequent cycle by the constant factor. The rate of the increase is greater for smaller M and r_m ; it equals to 1.59 [the same as the experimental data in Fig. 2(c)] for $M=20$ and $r_m=6.3$ in the case of 2D model with 30 pixel microarray element radius. The rate of g_i increase was always greater for 1D than for 2D model for any studied parameters M and r_m . Figure 5(b) represents the calculated curves corresponding to $M=20$ $r_m=6.3$ (diamonds and squares for 2D and 1D model, respectively). The experimental C_T values were equal to 30 and 23 for planar and capillary arrays, respectively. For clear comparison of the experimental and theoretical values, the threshold line of $\log_2(g_i)=20.4$ was applied to theoretical curves in Fig. 5(b) to intercept the 2D model curve at $i=C_T=30$ point. In this case theoretical C_T for 1D model equals 24 [see arrows in Fig. 5(b)] and is in good agreement with the experimentally shown value ($C_T=23$). It was shown experimentally that the double increase in melting time virtually inhibited PCR. To verify this finding the M value for the 2D model was increased from 20 to 40. In qualitative agreement with the experimental data, this increase in melting time has led to the increase in C_T from 30 to 37.5, although in the experiment this effect was stronger. For $M=7$ the model predicts $C_T=25$, while in experiment the decrease of C_T was by 1 cycle only. This dis-

FIG. 5. The results of numerical modeling. (a) Model with melting stage only. Cross section of PCR signal for different melting stage diffusion distances (r_m). (b) Model with melting stage only. Simulated PCR curves at exponential phase for $r_m=6.3$, microarray element radius $R=30$. Black diamonds: 2D model (planar array) melting stage duration $M=20$; gray squares: 1D model (capillary array) for $M=20$; white triangles: 2D model for $M=40$. Arrows indicate the way of C_T values calculation for 1D and 2D models with $M=40$ under condition corresponding to $C_T=30$ for the 2D model with $M=20$. (c) Model with melting stage only. C_T variation across the array element for 2D (solid diamonds) and 1D (open squares) models. (d) Model with three durations ($r_m=6.6$, $r_a=0.37$, and $h=0.09$). C_T variation across the microarray element. Black diamonds: $M=20$, $A=45$, and 2D model. Gray squares: $M=20$, $A=22$, and 2D model. White triangles: $M=20$, $A=45$, and 1D model.

agreement is discussed in more details in Sec. IV. The variation of C_T across the array element is given in Fig. 5(c). Similar to the experimental data [Fig. 4(a)], the variation was stronger for 2D than for the 1D model.

F. Numerical model with three PCR stages

To take into account the duration of the annealing and elongation stages, some additional parameters should be introduced into the model. These include durations of annealing and elongation stages, A and E , respectively, diffusion distances per 1 s of annealing and elongation stages r_a and r_e , respectively, and two hybridization parameters of annealing stage, h_1 and h_2 . The h_1 and $h_2 \in [0;1]$ are the portions of free PCR product and extended forward primers, respectively, hybridized with corresponding primers per 1 s of annealing stage. Here we assume the pseudofirst order of hybridization reaction due to the excess of primers over PCR products. The detailed description of the model could be found in the supplementary material (Ref. 27).

The model was executed with $h_1=h_2=h$, ranging from 0.02 to 0.2; r_a ranging from 0 to 3; r_m ranging from 3 to 8 with $M=20$, $A=45$; $M=20$, $A=22$; $M=40$, $A=45$ for 2D model and $M=20$, $A=45$ for 1D model. The results were found to be virtually independent on r_e and E ($\Delta C_T < 0.1$), ranging from 0.02 to 3 and from 10 to 40, respectively. Therefore, the elongation stage parameters are excluded in the following consideration for the reasons of simplicity.

The double reduction of annealing time resulted in slower growth of PCR signal for any

studied combination of parameters r_m , r_a , and h . Particularly, for $r_m=6.6$, $r_a=0.37$, and $h=0.09$ the increase in C_T was 3.7 cycles and the amplification factor was 1.57, close to experimental data 4 and 1.59, respectively.

To examine the variation of C_T across the array element, the ΔC_T was plotted relative to the center of the microarray element [$r_m=6.6$, $r_a=0.37$, and $h=0.09$, Fig. 5(d)]. Similar to experimental data, the shorter annealing stage resulted in the stronger change in ΔC_T across the array element, while in the 1D model the change in ΔC_T was weaker than in the 2D model (for comparison, see experimental data in Fig. 4).

The more detailed data with different combinations of r_m , r_a , and h and estimations of their acceptable ranges are given in supplementary materials (Ref. 27). Also, the effect of proximity of reaction chamber wall was studied. It was found that SP-PCR is weakly affected if the diameter of reaction chamber exceeds the diameter of gel pad more than twice, while the smaller reaction chamber leads to significantly more efficient SP-PCR. The efficiency reaches its theoretical maximum when the reaction chamber diameter approaches the diameter of gel pad. However, in practice the priming efficiency of free and immobilized primers should be taken into account.

IV. DISCUSSION

Experiments demonstrate the stronger variations of C_T across the microarray element than the numerical model does [Fig. 4(a) versus Fig. 5(c)]. This might be due to the convex shape of microarray element that resulted from the droplet polymerization. The gel layer is thicker at the center while at the border, its thickness reaches 0. As the model does not account for this change, it predicts the lower C_T variations.

Interestingly, the numerical modeling predicts the stronger signal variation across the microarray element for the lower r_m , i.e., for weaker diffusion [Fig. 5(a)]. This seems to be contrary to intuition: If the effect is caused by diffusion, it should gradually disappear with diffusion decrease. In fact, it disappears but in unusual manner: For the lower r_m , the cross section assumes its stationary shape later. Prior to assuming the stationary shape, the signal variation is lower than in stationary condition. However, for the stationary shape the variation should be stronger for the lower r_m . Indeed, the stationary condition implies (for the simple model) the balance between the broadening of cross-sectional curve caused by diffusion and the narrowing caused by new product synthesis. The broadening is stronger for the narrow shapes (because of high product gradient) and for high r_m . That is, a narrow shape for a low r_m [Fig. 5(a), opened squares] could give the same degree of broadening per melting stage as a broad shape for a high r_m [Fig. 5(a), solid squares].

The $r_m=6.3$ pixel=63 μm was estimated from numerical modeling. It is interesting to compare this value to earlier published data for diffusion coefficient D using the formula $r_m=2(D \cdot 1 \text{ s})^{1/2}$. For 20-base oligonucleotide at 20 °C the $D=1.52 \times 10^{-6}$ cm^2/s .²⁸ The empirical relationship $D \propto N^{-0.67}$ for N -base ds-DNA (Ref. 29) was successfully applied to single stranded oligonucleotides.³⁰ Using this relationship we can estimate $D=3.25 \times 10^{-7}$ cm^2/s for 200-base single stranded DNA free of any secondary structure. To extrapolate this value to 94 °C, we use the Stokes–Einstein relation $D=(kT)/(6\pi\eta r)$, where k is the Boltzmann constant, T is the absolute temperature, r is the effective radius, and η is the dynamic viscosity (1×10^{-3} N s/m² for water at 20 °C and 0.3×10^{-3} N s/m² for 94 °C). This gives $D=1.36 \times 10^{-6}$ cm^2/s for 200-base single stranded DNA at 94 °C, i.e., $r_m=23$ $\mu\text{m}=2.3$ pixel. This value has the same order of magnitude as the distance estimated from numerical modeling. This concordance is reasonable taking into account the potential inaccuracies due to extrapolation from 20 to 200 nucleotides and from 20 to 94 °C, as well as the model approximations.

Earlier it was demonstrated²⁰ for Actb that the C_T s for 600, 450, and 250 μm were 26, 30, and 39 respectively. Application of a numerical model with $r_m=6.3$ to the level of 26th cycle [similar to Fig. 5(b)] gives $C_T=30$ and 47 for the spot radii 23 and 13, respectively (data not shown). However $r_m=5.7$ (6.3 minus one standard deviation) with spot radius 14 gives $C_T=41$, compatible with the experimental data. The numerical modeling indicates that the outcome of PCR on small spots is vulnerable to subtle deviations of experimental parameters. It suggests once again that the small spots should be avoided for the reasons of reproducibility.

The most significant disagreement between numerical model and experiment is related to variations of melting stage duration. First, the double increase in the melting time virtually inhibited PCR, while the model predicts the C_T increase by 7.5 cycles. This discrepancy could be due to thermal inertia of heating device and to the neglected third dimension in the model. Indeed, the above difference could be increased up to 9.3 cycles (data not shown) if the thermal inertia is supposed to steal 5 s from the melting stage. The third dimension provides the additional room for PCR product diffusion rendering diffusion-caused effects stronger, thereby additionally increasing the C_T difference.

Second, the threefold decrease in melting time (from 20 to 7 s) resulted in insignificant change in C_T , while the model predicts its decrease by 5 cycles. This could be attributed to the thermal inertia of a heating system and (consequently or independently) the incomplete DNA strands separation. The latter is important for the successful SP-PCR as the incompletely separated DNA strand cannot diffuse and anneal to the solid phase primer. The complete separation of DNA strands implies the unwinding. In particular, complete unwinding of 10^5 bp DNA strands takes 10^2 – 10^3 s.³¹ The separation rate constants were found to be four to five times lower for T2 phage than for T7 phage,³² while their genome sizes are about 160 and 40 kbp, respectively. Therefore the assumed linear dependence of strand separation time on the DNA length could give the strand separation time around 1 s or below for 200 bp DNA. However, this time should be longer for the gel-immobilized DNA as the DNA attachment and the presence of gel polymer hinder the efficient strand unwinding. Noteworthy, this makes more the amplification of short fragments favorable, which often include nonspecific by-products of PCR. Therefore, the decrease in melting time should not be used as a means of SP-PCR improvement.

The most favorable way of SP-PCR efficiency improvement is the spatial restriction of diffusion by means of imposing physical obstacles (e.g., a capillary wall). It provides some additional opportunities. Particularly, it allows easy manipulations with the PCR solution. Earlier it was demonstrated that a capillary array of glass beads with immobilized oligonucleotides provides a superior nucleic acids hybridization.^{33,34} This was achieved by means of gradual pumping of hybridization solution through the capillary. These advantages could be helpful at prehybridization phase and annealing stage of SP-PCR; however, they are related to substantial engineering challenge and therefore go beyond scope of current investigation.

Also the modeling provides an estimation of geometrical proportions necessary for efficient restriction of the PCR product diffusion, particularly the reaction chamber diameter and the size of microarray element. The presented mathematical model could be easily expanded to the actual three-dimensional shape of microarray element and accounting the difference of diffusion coefficients inside and outside the gel pad. This could result in even better concordance with experimental data. Also microarray with complex geometry or with powered pumping of the PCR solution could be modeled in frame of proposed approach.

V. CONCLUSION

We demonstrated that the diffusion of the PCR product out of the microarray element is a key factor responsible for the difference between SP-PCR and traditional liquid phase reaction. The diffusion at melting stage is particularly important. The rate of PCR signal growth could be improved by imposing of physical restriction of reaction volume in the vicinity of microarray element (e.g., by a capillary wall). The obtained results could be useful for further development of SP-PCR applications in microarray technology.

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