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Arrayed primer extension on *in situ* synthesized 5′ → 3′ oligonucleotides in microchannels

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Abstract

Arrays of oligonucleotides synthesized in the 5′ → 3′ direction have potential benefit in several areas of life sciences research because the free 3′-end can be modified by enzymatic reactions. A Geniom One instrument (febit biomed GmbH, Germany), with integrated chip fabrication, multiplex primer extension, fluorescence imaging, and data analysis, was evaluated for studies of genomic variations. Microchannels used for the array synthesis in Geniom One were not optimized before for the APEX method and, as preliminary experiments demonstrated in this study, the signals were strongly affected by the speed of the process inside reaction channels. Using the two-compartment model (TCM), target binding to feature were quantitatively analyzed, revealing profound mass-transport limitations in the observed kinetics and enabling us to draw a series of physicochemical conclusions of the optimal set-up for the APEX reaction. Some kinetically relevant parameters such as target concentration, reaction time, and temperature were comprehensively analyzed. Finally, we applied the arrays and methods in a proof-of-principle experiment where 36 individuals were typed with 900 oligonucleotide probes (sense and antisense primers for 450 markers), using the ABCR gene as a test system. A new DNA analysis method for studies of genomic variation was developed using this all-in-one platform.

Introduction

Oligonucleotide microarrays are a powerful technological platform for large-scale screening of common genetic variations and disease-causing mutations [1–4]. The aim of this study was to establish the arrayed primer extension (APEX) reaction [5] on an *in situ* synthesized oligonucleotide microarray, with unconventional 5′ → 3′ oligonucleotide arrays, using the Geniom One platform (febit biomed GmbH, Germany), and to test its usefulness for designing an oligonucleotide array for large-scale applications.

Typically, 10–30% of oligonucleotide primers need to be redesigned to convert all sites (mutations/SNPs) into functional assays [6,7]. This is a time-consuming (several orders of magnitude of new oligonucleotide primers) and costly process (some oligonucleotides will only be used a few times). With SNPs, this is not a major problem because there are a large number to choose from without loss of genetic information. However, with defined mutations the situation is more complex and usually new primers will be needed with modified properties or further modifications. The maskless

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light-directed *in situ* microarray synthesis technology platform is a fast and cost-effective alternative for array development reducing the time needed for ordering new oligonucleotides and associated costs. Although this platform is not well suited for large-scale genomic association studies, it is ideal for analytical assays and array development.

One of the most important advantages of *in situ* oligonucleotide synthesis relative to prespotted arrays is its great flexibility of microarray design [8,9]. Tens of thousands of oligonucleotides can be synthesized simultaneously on a chip at relatively low cost, typically overnight. Because of this, testing of oligonucleotides in APEX reactions and chip redesign is greatly simplified. The *in situ* synthesized oligonucleotide arrays result in more uniform oligonucleotide attachment and can be produced at a much higher density [10]. Several methods for the *in situ* synthesis of oligonucleotide microarrays have been reported [1,11–13]. Among them, the method of mask-directed *in situ* parallel synthesis using a photolabile protecting group [1] has been successfully used to manufacture high-density microarrays in both the $3' \rightarrow 5'$ and $5' \rightarrow 3'$ directions.

The Geniom One instrument contains *in situ* DNA synthesis, reaction and detection units within one instrument allowing oligonucleotide synthesis, APEX reaction, washing and signal detection to be carried out in the same microchannels. The three-dimensional (3-D) microchannel cartridge is divided into eight individual subarrays each consisting of 6776 features that are independently available for different assays [14]. Its current version allows the synthesis of arrays of up to 48 000 oligonucleotide probes within microchannels of a microstructured biochip. Although the redesign and testing of new assays on the array is fast, it is not suitable for large-scale analysis where thousands of arrays are needed. Thus, we describe it as a personal genetic platform (PGP).

To benefit from this system, and recognizing the advantages of enzymatic reactions to analyze the variant nucleotides in the genome, the APEX reaction method was optimized and evaluated. The working principle of the APEX reaction is the DNA polymerase-based, template-dependent extension of oligonucleotide primers immobilized on a solid surface with fluorescently labeled dideoxynucleotide terminators [2,5]. Among many genotyping assays that have been developed over the past decade, methods based on primer extension (or single base extension) represent a simple and robust genotyping platform, which can be used in the analysis of known point mutations, deletions and insertions, and for the identification of unknown polymorphisms [4]. One commercial product embodying this is the APEX assay (www.asperbio.com). Here, the target nucleic acid sequence (patient PCR material) hybridizes perfectly to the oligonucleotide probe (APEX primer also defined as oligonucleotide primer). A DNA polymerase (Thermo Sequenase) specifically extends the 3′-end of the oligonucleotide primer with a fluorescently labeled dideoxynucleotide, complementary to the nucleotide at the DNA variable site [6].

Introducing the APEX method, which was optimized for prespotted oligonucleotide arrays on microscope slides to a microchannel biochip was a complex multi-parameter optimization problem, primarily due to strong mass-transport constraints and therefore slowed binding of the target to the adjacent oligonucleotide probes. Applying a two-compartment model (TCM), a mathematical tool enabling the analysis of mass-transport-dependent reactions [15–17], we could experimentally as well as theoretically demonstrate the effect of the biochip microchannel geometry on reaction kinetics. The TCM is a relatively simple model that dissects mathematically the parameters of mass-transport-dependent binding onto the ligand (oligonucleotide probe in present study), both mass transport from the bulk compartment to the reaction compartment and the subsequent binding step. This model has been originally proposed for the analysis of bimolecular interactions in the Biacore instruments and was newly modified and applied for kinetic analysis of antigen–antibody binding on microspots [18–20] on microslides. Despite the progress in microarray technology, the effects of diffusion and mass flux on the rate of the binding reaction at the microspot have been largely ignored over many years and mass-transport dependence on DNA microspot kinetics has not yet been experimentally investigated to our best knowledge. During the process we analyzed some important parameters such as hybridization temperature and time as we investigated kinetic aspects of the APEX reaction within the microchannel of the biochip. From our experience, this is the first quantitative experimental analysis of mass-transport dependence in oligonucleotide features.

Methods

To develop an APEX assay on an *in situ* synthesized oligonucleotide array, a test system was designed that allowed analysis of all basic parameters of the assay including temperature, time, viscosity, and template concentration. A panel of 36 DNA samples was assessed for particular gene mutation sites ($n = 450$) using an oligonucleotide microarray, the genotyping microarray for the ATP-binding cassette (ABCR) gene [7] from Asper Biotech (http://www.asperbio.com). The microarray includes 433 disease-associated genetic variants and 17 common polymorphisms of the ABCR gene. Genetic variation in the ABCR (ABCA4) gene has been associated with five distinct retinal phenotypes, including Stargardt's disease/fundus flavimaculatus (STGD/FFM), cone-rod dystrophy (CRD), and age-related macular degeneration (AMD).

All diagnostically relevant variations in the assay were positioned in exonic regions; thus, primers were designed to amplify particularly all exons of ABCR gene as it is described elsewhere [7]. Normally, all mutations in one exon as one PCR product of 100–400 bp can be analyzed together. Note that if two mutations are closer than 50 bp (25 + 25 nt long APEX primers) in the genome, then they cannot be analyzed on the same microarray owing to the competition in annealing step. The amplification step was performed in 24 single PCR reactions. The option to multiplex amplification was not applied owing to the need to confirm all found mutations by Sanger Sequencing.

Mutation analysis was carried out by single base extension with dye terminators on the APEX primers. Although the APEX reaction typically uses a four-color base detection system [5,21], the Geniom One system that was used had only two channels that detected signals only from Cy3 and Cy5. For the detection of all four nucleotides in the same DNA sample two microarrays (subarrays) were used: the first array for the detection of Cy3-ddATP/Cy5-ddGTP and the second for Cy3ddTTP/Cy5-ddCTP. The *in situ* synthesis of oligonucleotide microarrays was performed according to the manufacturer's protocol (febit biomed GmbH, Germany).

Perfectly matching sense and antisense 25-mer oligonucleotide probes were synthesized *in situ* for APEX reaction in the microchannels. Mutation analysis was carried out by a single base extension complementary to the template with dye terminators using DNA polymerase (Thermo Sequenase).

DNA samples

At first, 36 anonymous individuals having the most diverse set of mutations and SNPs in their ABCR gene [7] were selected from the Asper Biotech (Tartu, Estonia) database. PCR products consisting of 24 PCR fragments corresponding to all exons of the ABCR gene were synthesized by Asper Biotech and used as templates in validation experiments.

Template preparation

PCR primers were provided by Asper Biotech Ltd. (Tartu, Estonia). The amplification mixture was prepared and distributed into 50 μl aliquots. The PCR mixture contained 5 μl 10 × PCR buffer [containing 200 mM Tris–HCl, pH 8.4, 500 mM KCl (Life Technologies)], 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP), 0.2 mM dTTP, 0.05 mM dUTP (Fermentas, Vilnius, Lithuania), 40 pmol of each primer, and 1 unit of *Taq* DNA polymerase (ABGene, Epson, UK).The individual PCR amplification reactions were performed using a PTC-200 instrument (MJ Research, Inc., Watertown, MA). An initial incubation at 94 °C for 5 °min was performed, followed by 34 amplification cycles, consisting of denaturation at 95 °C for 20 s, primer annealing at 60 °C for 30 s, and extension 72 °C for 30 s, followed by a final extension at 72 °C for 5 min.

PCR products were pooled and purified with the QIAquick PCR purification kit (Qiagen), according to the manufacturer's protocol. Fragmentation and functional inactivation of unincorporated dNTPs was achieved in a one-step reaction by adding 1/5 U of shrimp alkaline phosphatase (Amersham Biosciences Europe GmbH, Freiburg, Germany) and 1/5 U of thermolabile uracil *N*-glycosylase (Amersham Biosciences Europe GmbH) per single amplification product. The reaction was incubated at 37 °C for 1 h and was then used directly in primer extension reactions. Unlike the APEX reaction with four different dye terminators [2,5,6,22], for technical reasons, two combinations of Cy3 and Cy5 fluorescent molecule combinations were used (ddATP-Cy3, ddGTP-Cy3, ddTTP-Cy5 and ddCTP-Cy5). The sample was divided into two portions and hybridized in parallel in different microchannels (subarrays); in this way, all four nucleotides could be analyzed.

In situ synthesis of the oligonucleotide microarray

The maskless, light-activated synthesis of oligonucleotide probes in the 5′ → 3′ direction in all the channels of the microarray and the detection of fluorescence signals were performed with the Geniom One (febit biomed GmbH, Germany) according to the manufacturer's protocols. The APEX reaction was performed externally in a hybridization oven to guarantee a constant temperature for the incubation.

Two oligonucleotide probe replicates were synthesized for the detection of each selected SNP/mutation on both DNA strands. A set of 6776 oligonucleotide probes including replicates and control oligonucleotide probes were synthesized that exhibited similar hybridization and enzymatic single base extension reaction characteristics under standard conditions. The size of the oligonucleotide feature was 34 μm × 34 μm. Within the channel, there were three oligonucleotide probes per row and 180 per column. APEX primers were selected for each analysis site.

The sequence information as a base alignment was converted by software to a design scheme for the microarray format. The resulting oligomers were attached via their 5′-ends, while the 3′-termini were freely accessible for the DNA polymerase [14]. In addition to the assay-specific oligonucleotides, 320 control oligonucleotide probes were synthesized on each array. To check the quality of the APEX reaction, self-priming oligonucleotide probes were used.

For the detection of A, TTTAGCCTTAACGCCT**T**TGACGTCA
For the detection of C, TTTAGCCTTAACGCCT**G**TGACGTCA
For the detection of G, TTTAGCCTTAACGCCT**C**TGACGTCA
For the detection of T, TTTAGCCTTAACGCCT**A**TGACGTCA

These primers form hairpin structures and are extended simultaneously with the normal APEX primers with fluorescent dye-conjugated dideoxynucleotides without any external template.

Arrayed primer extension reactions

In each APEX reaction, 200 ng of the amplified product was used. The 20 μl primer extension reactions consisted of 10 μl of fragmented product, 4 U of Thermo Sequenase DNA polymerase (Amersham Biosciences Europe GmbH), 2 μl of Thermo Sequenase reaction buffer (260 mM Tris–HCl, pH 9.5, 65 mM $MgCl_2$) (Amersham Biosciences Europe GmbH), and 1 mM final concentration of each fluorescently labeled ddNTP (PerkinElmer Life and Analytical Sciences, Boston, Ma, USA). DNA in the buffer was denatured at 95 °C for 5 min. The enzyme and dye were immediately added to the other components and the whole mix was applied to prewarmed (54 °C) microchannels of the biochip. The reactions were incubated for 90 min, and then stopped by washing at 95 °C with MilliQ water. The microchannels were then filled with Acetonitrile and signals were detected. The APEX signals were detected with the inbuilt CCD camera of the Geniom One unit.

Scanning and data analysis

APEX signals were detected with the Geniom One unit. The time-dependent development of the signal intensities was analyzed using a two-compartment model [15–17] as described previously [19,20]. According to this solution, adapted for microspot kinetics, the initial development of signal intensity of a mass-transport-limited reaction is linear and can be approximated to the following simple expression (see [18,19,23]):

$$S(t) \approx S_{\max} v_0 t, \quad (1)$$

where $S(t)$is the current signal intensity (SU, signal units), $S_{\max}$ is the maximally attainable signal intensity (SU), ratio $S(t)/S_{\max}$ is normalized signal intensity, t is time (s), and v_0 is the initial binding reaction velocity (SU/s). v_0 comprises two components: $1/v_0 = 1/v_{\text{ideal}} + 1/v_{\text{m}}$, where $v_{\text{ideal}} = k_+ L_0$ is an ideal initial binding velocity, $v_{\text{m}} = k_{\text{m}} L_0 / S_{\max}$ is mass-transport contribution, k_+ is a constant of association rate ($(\text{Ms})^{-1}$), L_0 is an initial analyte concentration (M) and k_{m} is a constant of phenomenological mass transport (SU/(Ms)). In the case of mass-transport-limited binding, where $k_+ S_{\max} \gg k_{\text{m}}$, with $v_{\text{m}} \ll v_{\text{ideal}}$ and $v_0 \approx v_{\text{m}}$, the Eq. (1) can be transformed:

$$S(t) \approx k_{\text{m}} L_0 t \quad (2)$$

To define the extent of mass-transport restrictions in the used system and to extract the k_m value the Eq. (2) was fitted to the initial slope of binding curve as described previously [18–20]. For the purpose of simplicity, for every tested oligonucleotide probe experimental data were first normalized to obtain maximal signal intensity so that the resulting S_{max} values in all cases were equal to 1, and $S(t)$ on the plots below represents relative signal intensities varying from 0 to 1.

Results

8.1. Control experiments on the test array

Two oligonucleotide microarrays were used for each DNA sample (Figure 1). To design a chip for the subsequent optimization experiments, 40 of the 450 probes representing SNPs and mutations were chosen with the highest signal-to-noise ratios from 100 previously tested APEX experiments on prespotted oligonucleotide microarrays. These sequences were synthesized *in situ* within microchannels of the biochip in $5' \rightarrow 3'$ direction. In order to check oligonucleotide probes for self-priming the APEX reaction was first performed without template DNA. Four different self-priming control probes, synthesized in three replicates per array, demonstrated positive signals, which were essentially independent of the presence of DNA. This provided information on the specificity of the APEX reaction. All other oligonucleotide primers synthesized on the same array did not show fluorescence signals in these APEX control experiments.

In addition to APEX control experiments without the template, several experiments in order to optimize the enzyme(s) and reaction conditions were performed. To select the best reaction conditions, initial optimization experiments were performed using control DNA of known quality. All reactions were repeated 10 times. Detected fluorescence signal intensities in the Cy3 channel were twice as high as measured in Cy5 channel owing to the physical differences of fluorophores, optional differences in nucleotide incorporation as well as technical properties of the detection system.

Reaction temperature

Conducting APEX reactions under stringent conditions (i.e. high temperature) is important for the binding specificity of the target and therefore will increase the successful call rate and specificity of the analysis. Different temperatures for the APEX reaction (45, 50, 55, and 60 °C) were compared. The optimal temperature was found at 50 °C. Higher hybridization temperature (55 °C) resulted in a notably lower level of signal intensities, until there were essentially no signals at 60 °C (Fig. S1). As expected, signal intensities increased at the lowest tested temperature (45 °C), but owing to the unspecific APEX reactions it was accompanied by a number of false positive signals.

Assessment of mass-transport effects

The slowing effect of reaction rates by mass transport can strongly influence important performance parameters such as sensitivity,

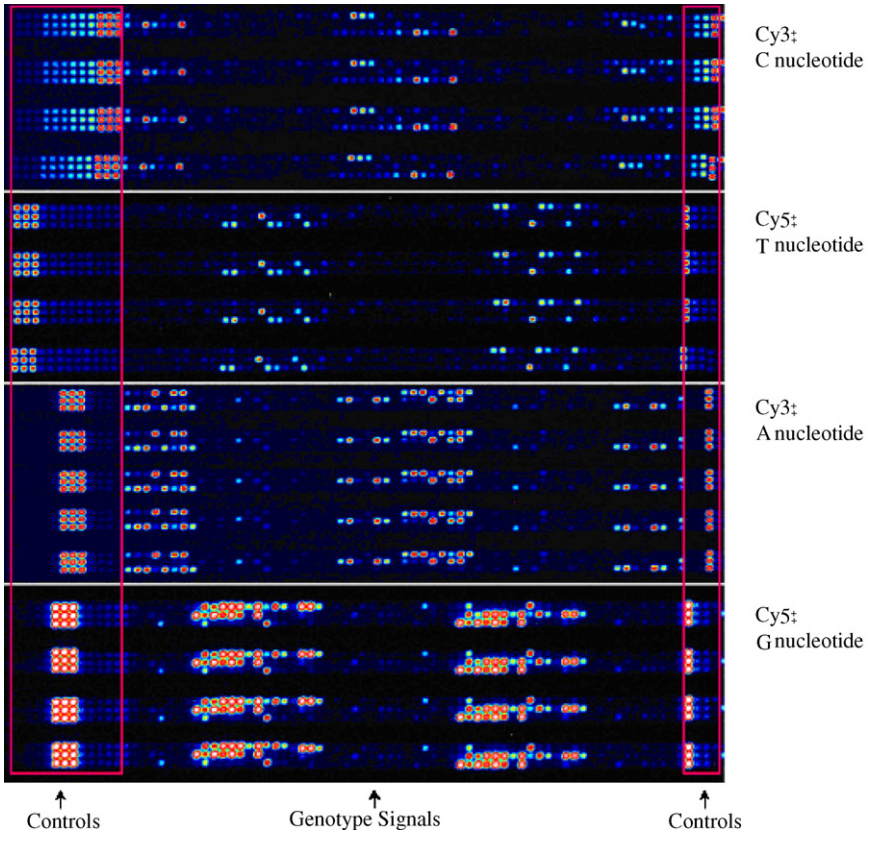

FIGURE 1

Images obtained by scanning a 'subarray' at two wavelengths (Cy-3 and Cy-5). The results from one individual genotyped by APEX are shown for the panel of 450 SNPs/mutations with oligonucleotide primers for both DNA strands. The obtained signals are reproduced with an artificial rainbow scale starting from blue for low signals until white for saturated signals.

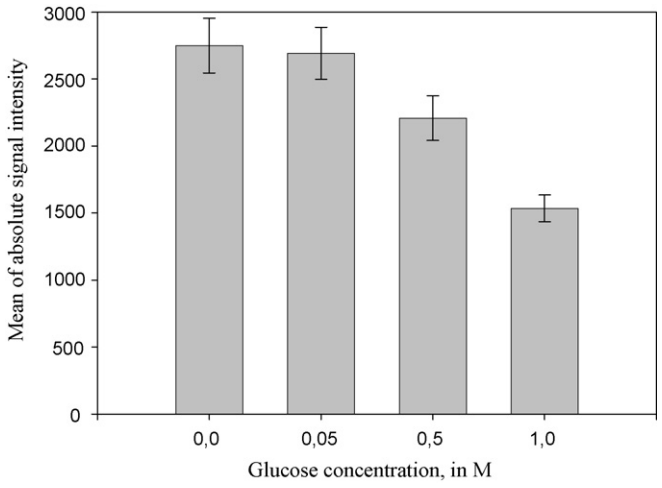

FIGURE 2

The dependence of mean signal intensities on glucose concentration in the incubation solution: 0 (reference), 0.01, 0.1 and 1 M. Different concentrations of glucose were added to change the viscosity of the solution.

reaction time, dynamic range and reproducibility [19]. The easiest way to examine this is to analyze the dependence between reaction rates and the viscosity of a solution. In case of mass-transport-limited reaction the viscosity is inversely proportional to the diffusion coefficient (Stokes-Einstein relationship) and thus to the reaction rate. Therefore, we varied the viscosity of the incubation solution by adding different concentrations of glucose (0, 0.01, 0.1 and 1 M). As expected, addition of low concentrations of glucose had little effect on signal intensities (Figure 2), but the overall mean signal intensity at 1 M glucose was nearly twice as low as in the solution without glucose (56%). The ratio of viscosity of glucose solutions to viscosity of water can be calculated from the volume fraction of the suspended glucose as indicated in the literature [24]. For 0.1 and 1 M we could obtain viscosity ratios of 1.05 and 1.5, respectively. These values matched well with the observed factors of signal intensity decrease at corresponding glucose concentrations, which reflect decrease of reaction rates with increased viscosity of the solution (Figure 2). From that, we can conclude that the diffusional constraints in the bulk solution represent the limiting factor for the most interactions in the microchannel.

Reproducibility

In trying to produce binding curves for a quantitative kinetic analysis (see below), we found that reproducibility was poor, which obliged us to examine the issue in more detail. Different oligonucleotide probes were synthesized in 11–12 replicates per tested condition and analyzed at different time points from 10 min to 12 h of incubation, with the concentration row of the corresponding PCR products (0.1, 0.5, 1, 2, and 10 ng/μl). The obtained signal intensities were analyzed as suggested previously [23] for an estimation of overall effects in complex microarrays. Specifically, individual signal intensities were first normalized per the mean of all signals at corresponding time points and the concentrations and CV%s from more than 40 of the normalized values were calculated over all tested oligonucleotide probes. Additionally, 48 positive controls per each tested condition were analyzed to obtain values for experimental variation in system.

While the observed variance in signal intensities seemed to be specific for particular oligonucleotide probes, the general trend was similar for all sequences. The highest variance in signal intensity was observed at low concentrations (0.1, 0.5, and 1 ng/μl) and short incubation time (up to 1 h). With increased target concentration, from 0.1 to 10 ng/μl, CV% values decreased by two to three times, and increased incubation time lead to a slight but significant improvement in CV% in the case of low concentration PCR products.

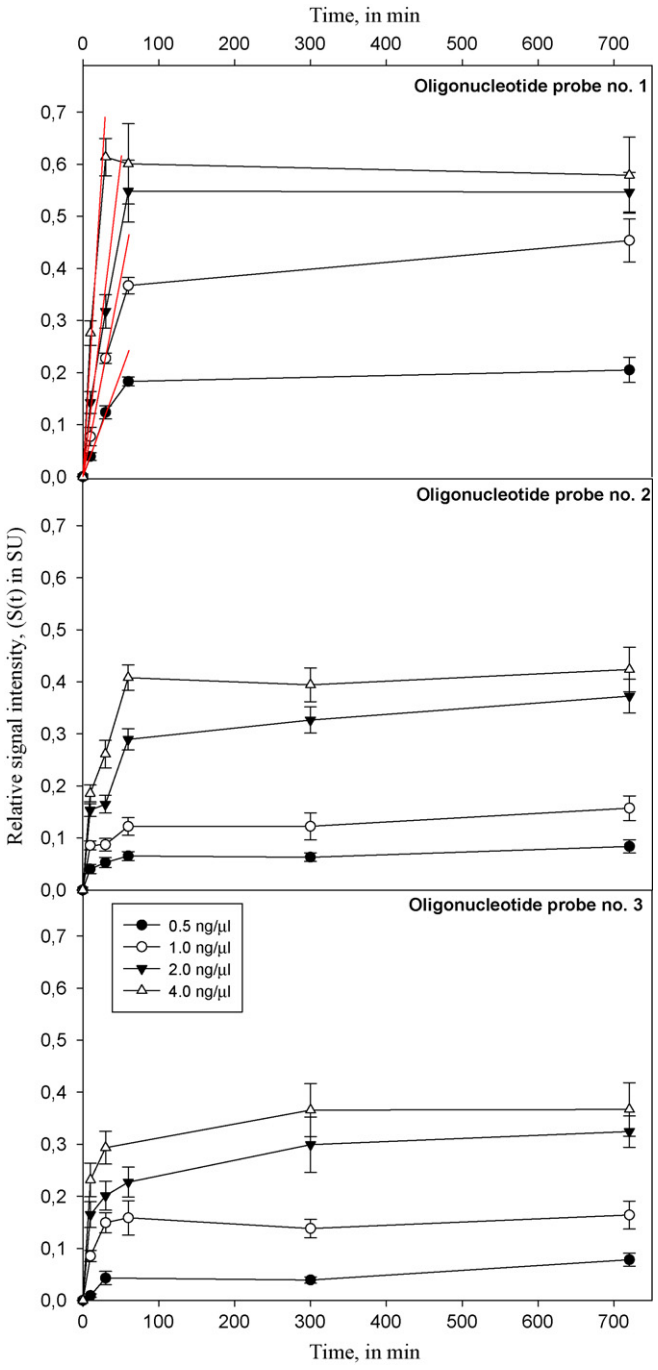

FIGURE 3

The progression of binding curves for 0.5, 1.0, 2.0 and 4.0 ng/μl amplicons concentrations at incubation times of 10, 30, 60, 300 and 720 min. The initial signal development was fitted to Eq. (2) (red lines). See Table 1 for the obtained parameters and other details.

TABLE 1

Experimental descriptors of the three quantitatively analyzed hybridization complexes

Oligo no.	Sequence of the probe	Target length (bp)	MW (kDa)	c_{Molar} in nM for 1 ng/μl	k_m (SU/Ms)	Maximal signals $S_{max-abdolut}$	k_d (10^9 M)
1	AGGCCCCTCATGCAGAATGGTGGTC	325	105.9	9.441	12154 ± 1880	12505 ± 915	~18
2	ACATGTTCTGGGCCGGAGAGGTATT	330	107.7	9.301	(13787 ± 4666)	35292 ± 2476	~45
3	GCTCACGATGACATTCCACAGCATG	407	132.7	7.530	(12971 ± 3170)	27838 ± 2776	~42

The equilibrium dissociation constants (k_d, M) were estimated by fitting the saturated signal intensities to the equation $S_\infty/S_{max}=L_0/(L_0 + k_d)$, where S_∞ is a signal intensity upon achievement of the thermodynamic equilibrium (in SU; see for details supplement). The phenomenological mass-transport constants (k_m, SU/Ms) were calculated using Eq. (2) (see text). The length, the molecular weight, and the total molar concentration of the target (c_{Molar}, M) in the reaction solution are obtained from known sequences of PCR products. Relative signal intensities ($S(t)$) were calculated upon normalization against maximal signals obtained ($S_{max-abdolut}$) and S_{max} values were set to 1.

Quantitative kinetic analysis: optimal PCR product concentration and incubation time

PCR product concentration and incubation time, as well as attainment of thermodynamic equilibrium by optimal choice of both parameters, are crucial factors in enabling improvements in the signal-to-noise ratio and reproducibility of the APEX reaction. In order to optimize these parameters, the progression binding curves for 0.5, 1.0, 2.0 and 4.0 ng/μl amplicon concentrations were analyzed at incubation time of 10, 30, 60, 300 and 720 min (Figure 3). Owing to considerable variability in signal intensities, as described above, lower concentrations of targets could not be analyzed. Ten replicates were synthesized per each oligonucleotide probe. In order to minimize the influence of competitive binding from neighboring probes, replicas within the same channel were positioned at the maximum distance from each other. All signal intensities were normalized against maximally attainable signals ($S_{max-absolut}$) for each oligonucleotide probe (Table 1), so that S_{max} was always equal to 1 and $S(t)$varied between 0 and 1. The estimation of maximal signals was done by fitting the dose-response curves to the following equation as shown in Fig. S2: $S_{max-absolute} = (L_0+k_d)/L_0S_\infty$, where S_∞ is absolute signal intensity upon achievement of the thermodynamic equilibrium. The initial linear phases of progression curves were fitted to Eq. (2). Additionally, converting the target concentrations into the molar form, by using known lengths of PCR products, we could also approximately estimate k_ds, the equilibrium dissociation constants in M, for the analyzed interaction of partner pairs. All the data for the three probes are summarized in Table 1.

The progression curves of only one of the tested probes (Oligo no. 1) could be perfectly fitted to Eq. (2) at all tested concentrations (Figure 3, Table 1). In the case of two other oligonucleotide probes (nos. 2 and 3), the initial linear phases were however too short for such fitting approach. To note exponential growth of signal intensities in mass-transport-limited as well as in ideal reaction-limited kinetics at $L_0 \ll k_d$ (4,7), as was observed for the both oligonucleotide pairs (see k_d s and tested concentrations in Table 1 and Figure 3), makes it difficult to differentiate visually between these two reaction types [18–20]. On the contrary, signal development at target concentrations in the limit $L_0 \gg k_d$, which is strongly linear if limited by mass transport, were not useful for quantitative kinetic analysis in this study because the saturation of signal intensities was attained too fast. Therefore, k_m values for both probes were calculated using Eq. (2) from signals at the earliest time point (10 min) and thus represent only approximate values (Table 1). As expected, all the obtained values were consistent with each other and indicated that, despite relatively low interaction affinities within the mid-nanomolar range, all analyzed interactions were slowed by the same mass-transport effect.

Performance of the developed genotyping system

The incubation conditions such as temperature and time were determined in the previous optimization step, thereafter an entire set of oligonucleotide probes (for sense and antisense strand) was synthesized for 450 SNPs and mutations resulting in 900 probes in total. These were synthesized in four replicates. First, four genomic DNA samples from individuals in our studied group were tested in order to validate the assay. Then, a blind experiment was run with 36 anonymous individuals to determine the quality of the assay and subsequently the results compared with those obtained by Asper Biotech Ltd. All found mutations were confirmed 100%. The results of four individuals out of 36 overlapped one to one with those produced by Asper Biotech, but for the rest (32 individuals) the call rate of SNPs for sense strand was 96% and for antisense 87% in respect of all variations. The total concordance of detection results (for mutations and SNPs together), estimated over 36 individuals, obtained in present study was 98% in comparison with data from Asper Biotech.

Discussion

The intention of this study was to set up the APEX reaction on the Geniom One platform, offering benefits over conventional, prespotted oligonucleotide microarrays in terms of cost, flexibility of design, stability of oligonucleotide probes and multiplex analysis. The primary technological problem was the novel microenvironment of the APEX reaction. The microchannels as the reaction vessels may slow down the reaction velocity due to the surface effects and viscosity of the reaction mix.

PCR amplification of 24 products flanking the regions of interest was performed by the protocol in the Methods section. The preparation of the target material for APEX comprises in addition fragmentation of PCR products. In other words, the fragmentation step of amplicons, obtained in the PCR, produces effectively short target sequences for the hybridization on an oligonucleotide probe attached to the surface with minimum steric hindrance [5]. In this way, the target length and sequence are independent of primer design that has to be therefore optimized and checked *in silico* for potential cross-hybridization only once. Consequently, in APEX method the PCR primer design and further redesign is not as crucial as with the allele-specific hybridization approach. Therefore, the APEX method is in addition to other various simplifications conveniently robust method for using it for the aforementioned purposes [22]. To date there is an option to over-

come the ever-existing multiplex PCR amplification problem by using universal primer in the amplification step that is well suited for APEX [25].

The APEX reaction occurs in two steps: target sequence hybridization with a primer, followed by an enzymatically catalyzed covalent binding of a conjugated dideoxynucleotide at the 3′-end of the oligonucleotide primer. Enzyme is in abundant excess in the reaction mixture and requires only a few seconds or less to incorporate dye labeled ddNTPs to all oligonucleotide primers on the chip (according to technical data provided by Amersham Biosciences the incorporation rate of dNTPs, for example, is more than 10 nmol in 30 min at 74 °C). Therefore, the enzymatic step cannot be the limiting factor in APEX reaction. The most relevant is the first hybridization step between the patient DNA (PCR product) and oligonucleotide probe in the microchannel. Since all analyzed interactions are limited by mass transport, the characteristic time of the exponential signal development can be simply depicted as $\tau_m \approx S_{max}/k_m k_d$ in the limit of $L_0 \ll k_d$ [18,19,26]. Depending on interaction of the annealing pair, half maximal signal intensities $S(t) = \alpha\, S_{max} L_0/(L_0 + k_d)$ with $\alpha = 0.5$) could be attained theoretically between 20–50 min ($t \approx 0.69\ \tau_m$; see also Table 1) and nearly saturated signals ($\alpha = 0.9$, $t \geq 2\ \tau_m$) need up to 2.5 h. This is in good agreement with obtained experimental results (Figure 3). However, the interaction affinities analyzed in this study are quite weak, while k_d values for DNA–DNA duplexes are usually much stronger [27], for example, a 20-mer oligonucleotide may have k_ds of 10^{-11} to 10^{-14} M, while longer DNA fragments may reach values of 10^{-10} to 10^{-30} M as measured in solution. This situation for the limit $L_0 \ll k_d$can be simulated (Fig. S3) using the k_m values obtained in this study and assuming slower or faster mass-transport conditions. According to this simulation for relatively slow diffusing components like long oligonucleotide fragments or high molecular weight proteins, one would need hundreds of hours to reach the saturation of signal intensities in the microchannel geometry in the case of high or middle affinity interactions. If $k_d \ll 10^{-10}$ M, saturation is not attainable for any partners interacting with an oligonucleotide probe in microchannels.

With increased L_0, signals develop linearly at L_0 close to k_d value, the time required to attain the thermodynamic equilibrium is significantly shortened (see Oligo no. 1 on Figure 3) and becomes inversely proportional to L_0 at $L_0 \ll k_d$ (Eq. (2)) [19,20]. Consequently, to attain maximal signal-to-noise ratios and best reproducibility, the concentration of PCR products has to be sufficiently high and the incubation time has to ensure the saturation of signal intensities for all probe-target sequences within the microchannel chip. More as 1 nM and at least 1 h of incubation time are these experimentally obtained parameters in system we developed.

In addition to the recently published studies on TCM-based analysis of protein microspot kinetics [18,19,23], the present report demonstrates the applicability of the developed model in the case of DNA oligonucleotide microarrays. It should be emphasized that the main advantages of the TCM are the options to analyze quantitatively the performance of a particular system without involving complicating prediction parameters like the density of binding sites, diffusion constant, stirring velocity or reaction mechanism (as long as k_m = const) as well as to simulate a wide range experimental conditions that are difficult or even impossible to analyze in the practical context.

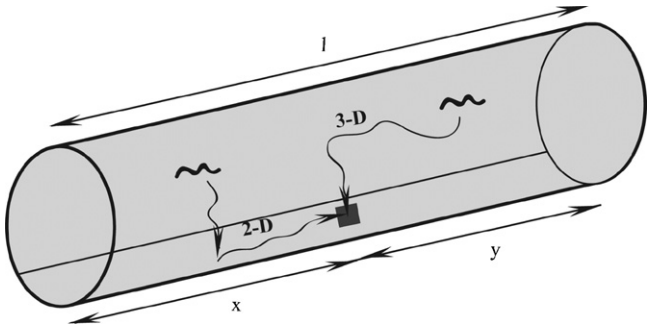

FIGURE 4

The schematical presentation of the target binding to the oligonucleotide feature within the microchannels. 3-D binding by the direct diffusion from the bulk solution; 2-D binding by the two-step reaction mechanism: adsorption on surface followed by the 2-D search for the binding sites along the surface. *x* and *y* are dimensionless coordinates of an oligonucleotide feature within the microchannel, *l* is the length of the microchannel in cm (see also Eqs. (3) and (4)).

It is of great interest for microchannel-based approaches to understand the potential nature of the mass-transport limitations observed above. Considering only diffusion in the solution, the motion of ligand molecules diagonally to the channel could be ignored because of the highly disparate dimensions of the microchannel system used (110 μm radius and about 9 cm length) (Figure 4). In such case, one can assume that ligand molecules that reach the proximity of the oligonucleotide feature are immediately bound and the mean reaction time for horizontal, 1-D transport rate along the channel is (for 1-D diffusion in an incubation chamber see [26])

$$\tau_0 = \frac{(x^2 + y^2)l^2}{6D}, \tag{3}$$

where D is the diffusion coefficient in dm^2/s, l is the length of the channel in dm, and $(x^2 + y^2)/2$ is a dimensionless geometrical factor describing the position of the oligonucleotide feature within the channel, with $x^2 + y^2 = l$. From Eq. (3), one can derive the association rate constant,

$$k'_m = \frac{6Dr_c^2}{(x^2 + y^2)lR^2\rho}, \tag{4}$$

where k'_m is the association rate constant for 1-D diffusion in 1/Ms, r_c is the radius of the channel in dm, R is the oligonucleotide feature radius in dm and ρ is the surface density of oligonucleotide probes in mol/dm^2. Interestingly, this association rate strongly depends on the geometric parameters of microchannel and it can be quadratically increased with increased channel radius. At known values, r_c=111μm, l = 9 cm, $R \approx 17$ μm, assuming that ρ and D are both in the range of 10^{-11} mol/cm^2 and 10^{-6} cm^2/s respectively, one could obtain values comparable to those obtained experimentally in the current study for k_m (see Table 1). Consequently, such transport mechanism can contribute to the overall reaction rates. To estimate whether channel geometry could impact reaction rates alone, the Eq. (4) can be compared with association rate constant for a hemispherical drop that is placed centrally over the same oligonucleotide spot ($k_s = 4D/\pi R\rho$, see [19,20,26]). In our case, the diffusion dependent reaction would be slowed approximately by ten to twenty times, depending on the oligonucleotide probe position within the

microchannel. Moreover, the optimal combination of oligonucleotide feature size and microchannel geometry provides maximal reaction rates ($k_s/k'_m = 2(x^2 + y^2)lR/3\pi r_c^2 = 1$, e.g. at $l = 9$ cm, $R \approx 17$ μm, r_c in the range of 0.5 mm). Outside of this optimum an active mixing (or pumping) of the reaction mix becomes extremely important in order to maintain the appropriate reaction conditions.

Furthermore, a very low sample amount to surface ratio in the microchannel geometry would in addition increase its strong non-specific adsorption from the bulk solution [19,20]. Even if the initial PCR product concentration depletes and the subsequent binding from the bulk solution weakens as described by Eq. (4), it may still have a strong positive effect. A two-stage mechanism, non-specific adsorption of target on surface and subsequent 2-D search for probes, has been classically considered in DNA microarray technology [19,28–30] as a mass flux mechanism that has a strong influence on overall reaction rates. The highest effect is attainable with short oligonucleotides of only few dozen nucleotides [30]. As the fragmentation of relatively long and slowly diffusing PCR products has been performed in this study, it is indispensable for the performance of the established microchannel approach.

Improvement of signal-to-noise ratio and reproducibility is mainly a choice of the optimal target concentration and incubation time. Increased CV-values are correlated with the decreased target concentration and shorter incubation time. Statistical fluctuation for various interacting partners on oligonucleotide probe and experimental error (see oligonucleotide probes on Figure 3) may be responsible for lower reproducibility. Similar correlations have been observed also on antibody microarrays (data not published).

In contrast to the allele-specific hybridization genotyping approach [31], the reaction temperature in the APEX reaction has to be higher owing to the enzymatic processing specificity. Whereas higher temperatures are reported to increase k_d of solid phase hybridizations [32], this consequently leads to lower signal intensities likewise observed in the present study. Optimal hybridization temperature in the APEX reaction is therefore a compromise between the optimal enzymatic activity and maximally attainable signal intensities.

In summary, the present study has demonstrated the design of a functional assay for both mutational and SNP analysis by the APEX reaction, using an *in situ* synthesized oligonucleotide microarray in reverse direction (5′ → 3′). The Geniom One platform used in this study contains an integrated microarray fabrication and detection system and enables the production of up to 48 000 oligonucleotide probes. Parameters affecting the success of the APEX reaction such as hybridization temperature, PCR product concentration, incubation time and variability of signal intensities were analyzed and optimized in this study. The main hurdle in the process of optimization is the novel microenvironment of microstructured biochip for the APEX reaction—the microchannel reaction carrier represents an additional barrier for diffusional transport of target oligonucleotide to oligonucleotide probes. Applying the TCM, we achieved success with a quantitative analysis of mass transport depending on the interactions within the microchannel system. This has enabled us to depict the potential reaction mechanism and to draw the conclusions from this hybridization geometry for the performance of the APEX reaction. Obtained genotyping results were compared with data from a prespotted oligonucleotide microarray. In conclusion, the accuracy, sensitivity and reproducibility of the established microarray system within the microchannel reaction carrier were comparable and consistent with a planar genotyping platform.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbt.2008.08.001.

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