





**JANNE PULLAT**

Design, functionalization and  
application of an *in situ* synthesized  
oligonucleotide microarray



TARTU UNIVERSITY  
PRESS

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## LIST OF ORIGINAL PAPERS

Current thesis is based on the following original publications which will be by their Roman numerals:

- I. **Pullat, J.**, Fleisher, R., Becker, N., Beier, M., Metspalu, A., Hoheisel, J.D. 2007. Optimization of candidate-gene SNP-genotyping by flexible oligonucleotide microarrays; analyzing variations in immune regulator genes of hay-fever samples. *BMC Genomics* Aug 17, 8, 282–294.
- II. **Pullat, J.**, Kusnezow, W., Jaakson, K., Beier, M., Hoheisel, J.D., Metspalu, A. 2008. Arrayed Primer Extension on *in situ* Synthesized 5'→3' Oligonucleotides in Microchannels, *New Biotechnology* (2008), August 14, PMID: 18786662
- III. **Pullat, J.**, Metspalu, A. 2008. Arrayed primer extension reaction for genotyping on oligonucleotide microarray. In *Methods in Molecular Biology: Prenatal Diagnosis: Preface*. Ed by S. Hahn, L.G. Jackson. Prenatal Diagnosis 435, Series: *Methods in Molecular Biology*. ISBN: 978-1-58829-803-4, Humana Press. Vol. 444, 161–167.
- IV. Bauer, A., Beckmann, B., Busold, C., Brandt, O., Kusnezow, W., **Pullat, J.**, Aign, V., Fellenberg, K., Fleischer, R., Jacob, A., Frohme, M., Hoheisel, J.D. 2003. Use of complex DNA- and antibody-microarrays as tools in functional analyses. *Comparative and Functional Genomics*, 4, 520–524.

My contributions to the articles are as follows:

- Ref. I I designed the assay, performed various experiments, analyzed the results, and was responsible for drafting the manuscript;
- Ref. II I conducted this study, designed the assay, performed all of the experiments, analyzed all of the results, and was responsible for drafting the manuscript;
- Ref. III I performed the APEX experiments and was responsible for drafting the manuscript;
- Ref. IV I conducted this study, designed the assay, performed all of the experiments, and analyzed all of the results.

## LIST OF ABBREVIATIONS

APEX	Arrayed Primer Extension Reaction
ASO	Allele-Specific Oligoprobe
A/T/G/C	Adenine/Thymine/Guanine/Cytosine
bp/kb/Mb	Base pair / kilo (10 <sup>3</sup> ) base pair/ mega (10 <sup>6</sup> ) base pairs
CNV	Copy Number Variation
CV	Coefficient of Variance (%)
ddNTP	Dideoxyribonucleotide Triphosphate
ddNMP	Dideoxynucleoside Monophosphate
DASH	Dynamic Allele Specific Hybridization
DCA	Dichloroacetic Acid
DMD	Digital micromirror device
DMT	5'-O-4,4'-Dimethoxytrityl
dsDNA	Double Stranded Deoxyribonucleic Acid
FRET	Fluorescence Resonance Energy Transfer
HPV	Human Papilloma Virus
HSIL	High Grade Squamous Intraepithelial Lesions
LD	Linkage Disequilibrium
L1	HPV gene, codes the major capsid proteins
MALDI-TOF	Matrix-assisted Laser Desorption/Ionization Mass Spectrometry
MeNPOC	$\alpha$ -Methyl-2-Nitropiperonyloxycarbonyl
mRNA	Messenger RNA
NPPOC	3'-Nitrophenylpropyloxycarbonyl
nt	Nucleotide
OH <sup>-</sup>	Hydroxy group
OLA	Oligonucleotide Ligation Assay
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PGA	Photogenerated Acid
PLPG	Photolabile Protecting Group
PM/MM	Perfect Match/ Mismatch Oligonucleotide Probes
RT-PCR	Real Time Polymerase Chain Reaction
SBE	Single Base Extension
SBH	Sequencing by Hybridization
SNP	Single Nucleotide Polymorphism
STR	Short Tandem Repeat
TCM	Two-Compartment Model
tiff	Tagged Image File Format, filename extension
txt	Text file, filename extension
WGG	Whole Genome Genotyping
3D-view	3-Dimensional View
5'-O-(MeO) <sub>2</sub> Tr	5'-O-dimethoxytrityl

## INTRODUCTION

Each disease and human trait has a genetic component. Due to the recent rapid advances in genomics and bioinformatics, we are beginning to understand how genetic variation influences human health. Both sequencing and genotyping efforts produce massive amounts of information, including the identification of genetic markers involved in disease development. However, these population-based and/or case-control studies are not designed or suited to analyze particular genetic variants in individual patients. The current Ph.D. thesis is devoted to increasing understanding of the technology available for analyzing polymorphic variations and mutations in the human genome, in order to enable the development of diagnostic tests using microarray platforms in near future.

In molecular diagnostics, microarray-based assays enable the flexible identification of disease-associated SNPs, mutations (including indels), gene copy numbers, viruses, and other pathogens. These assays are usually quite robust, highly parallel, and are increasingly used in diagnostics laboratories. Microarray technology has come a long way technically since its introduction in the 1990-s, when it was initially designed for large-scale mapping and DNA sequencing. However, the basic idea – small size, highly parallel, robust in application, and economical in production – could be readily adapted to a large variety of applications. On their own or in combination with other methods, microarrays have opened many new avenues in biological research and are used along with DNA analysis in transcriptomics, proteomics, etc. There were two dominant oligonucleotide microarray techniques in use when this project was started: the spotting method and Affymetrix-like microarrays manufactured using light projection by semiconductor-like masks.

With the photolithographic maskless synthesis of oligonucleotide microarrays assisted by micromirrors (i.e., as are used in slide beamers), microarray production has changed remarkably. Bench-top instruments have been developed that enable the *in situ* parallel synthesis of many oligoprobe arrays, each with thousands of features. Furthermore, efforts towards downscaling the consumption of DNA samples and other chemicals have led to the development of microfluidic handling structures, such as micro-sized channels. These “lab-on-a-chip” devices integrate one or several experimental functions on a single microarray. Among the many differences between slide-based arrays and microchannel devices, the influence of the reaction vessel geometry is one of the most important. The main technological hurdle towards the development of a “lab-on-a-chip” is the novel reaction microenvironment; using microchannels as reaction vessels may slow down the reaction velocity, due to surface effects and the viscosity of the reaction mix.

The basic aim of the present study was to use one of the first microarray instruments of its kind (Geniom One, febit biomed GmbH, Heidelberg, Germany) to determine the chemical, physical, and enzymatic parameters for oligonucleotide probe array synthesis using microchannels. Two types of reactions were used to model real diagnostic tests.

# I. OVERVIEW OF LITERATURE

## I.1. DNA analysis

Analytical tool development has greatly benefitted from the development of technologies (such as microarrays) that allow efficient, reproducible multiplex genotyping at the single nucleotide level at low cost (Pastinen et al. 1997). Markers used in these technologies include anything that can be measured reliably (Baker 2006): SNPs, point mutations, DNA methylation patterns, gene expression patterns, or protein presence/distribution. In addition, copy number variation (CNV) (Macconail et al. 2007; Thomas et al. 2007) across individuals in the human population (Feuk et al. 2006) may be important in the study of various diseases (Macconail et al. 2007). Microarrays are classified on the basis of their arrayed material, such as DNA, nucleic acids, proteins, antibodies, carbohydrates, tissues, or cells. Additionally, they can be grouped by the solid support used (glass, silicon, etc.) or by the type of analytical reaction performed (hybridization only or hybridization plus enzyme step, etc.).

The large-scale genotyping of human DNAs is one effective way to determine disease susceptibility among individuals. Studies of common diseases fall into two broad categories: family-based linkage studies across the entire genome using small sets of 400–1000 short tandem repeats (STR) markers, and genome-wide association studies using high-density SNP arrays with 100,000–1,000,000 single nucleotide polymorphisms (SNPs) plus an equal number of non-polymorphic SNPs for CNV analysis. In addition, an intermediate step involves the use of a candidate gene approach, where a given set of genes are tested in a case-control study design using SNP genotyping (Syvanen 2005). Recently, advanced high-throughput experimental techniques have enabled whole genome genotyping (WGG) to determine hundreds of thousands of SNP markers on a genome-wide scale (Gunderson et al. 2005; Steemers and Gunderson 2007).

SNP markers are the most common targets for high-throughput genotyping. In order to describe the genome and take advantage of the linkage disequilibrium, there is a certain freedom in choosing the best-performing SNPs without losing genetic information at a specific locus. In routine diagnostics, analysis of a particular marker, such as a single point mutation or other type of sequence change, is often necessary. Therefore, the test must perform well at DNA sequence sites close to the mutations.

In principle, DNA microarray technology represents the most powerful tool possible for SNP analysis, given its flexibility and speed in designing diagnostically-relevant assays. Despite numerous advancements in microarray development, there remain demands to improve the quality of DNA microarray preparations (Gao et al. 2004). Newly developed methods of high-throughput analysis strategies have moved away from classical formats (A del Campo 2005); the best example of this is the Illumina platform, which is based on microparticles but organized in an array format (Steemers and Gunderson 2007).

Current genotyping most often (but not always) involves discriminating allelic variants that differ by a single base (Wang et al. 2005). There are various methods and detection platforms that could be used for allelic discrimination. Most genotyping assays belong to one of four groups, based on molecular mechanisms: allele-specific hybridization, primer extension, oligonucleotide ligation, and invasive cleavage (Gut 2001; Sobrino et al. 2005; Syvanen 2005; Hoheisel 2006; Tost and Gut 2006). Most of these methods, however, are limited by their multiplexing capabilities.

## I.2. Microarray platforms

In research and diagnostics laboratories, the analysis tools routinely used can rapidly perform many different assays. Microarray technology, as an extension of microplate technology, is the most widespread platform currently used for nucleic acid analysis, because it enables the simultaneous analysis of large amounts of genomic material in a small format. Subsequent advances in microarray technology have concerned the flexibility and speed of assay development.

Genotyping-based microarray technologies are principally comprised of two reaction types: hybridization only and hybridization followed by an enzymatic reaction (e.g., Arrayed Primer Extension, APEX). The microarray-based analysis of DNA variations directly depends on the hybridization of a target DNA strand to its complementary oligonucleotide probe immobilized on the slide surface (Lockhart et al. 1996; Mir and Southern 1999; Southern et al. 1999; Tillib and Mirzabekov 2001). These analyses also directly depend on the complementary properties of the DNA, such that its single-strand sequence hybridizes specifically to the complementary probe (Wetmur and Davidson 1968; Wetmur 1976; Drmanac et al. 1989; Khrapko et al. 1989; Maskos and Southern 1992; Maskos and Southern 1992). Thus, this method involves immobilization of oligonucleotide probes in a microarray format, hybridization with a very large set of DNA PCR amplicons, labelling with fluorescence molecules, examination of the fluorescence pattern, and computation of DNA sequence variation.

The factors that critically determine the quality of microarray experimental results are determined by the characteristics of the different types of microarrays and their fabrication technologies. Six distinct technological platforms are currently used to manufacture oligonucleotide DNA microarrays.

- 1) **Spotting** uses spotting robots and presynthesized oligonucleotide probes, and is the most widely-used microarray platform (Chrisey et al. 1996);
- 2) ***In situ* synthesis by printing** uses Ink-jet or other printing technologies to deliver A, C, G, and T phosphoramidites to specific binding sites on the oligonucleotide probe matrix (Maskos and Southern 1992; Case-Green et al. 1998);

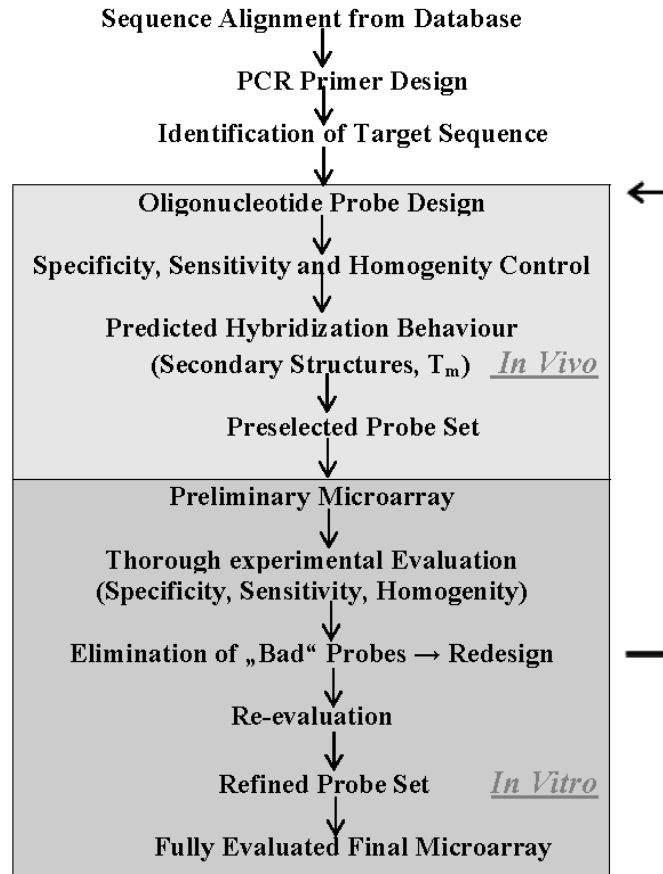
- 3) ***In situ* parallel synthesis exploiting electrogenerated acid** uses microarrays of individually addressable electro-reaction cells (Egeland et al. 2002);
- 4) **Maskless photogenerated acid (PGA) – controlled *in situ* synthesis** uses digital photolithography and phosphoramidites (Singh-Gasson et al. 1999);
- 5) **Mask-directed *in situ* parallel synthesis utilizing photolabile protecting groups (PLPGs)** uses photomask and PLPGs on phosphoramidite monomers (Fodor et al. 1991; McGall and Fidanza 2001); and
- 6) **Maskless *in situ* parallel synthesis utilizing PLPGs** uses digital photolithography and PLPGs on phosphoramidite monomers (Beier and Hoheisel 1999).

Critical variables that can influence the quality of experimental results depend on technological specifications, including:

- 1) the **synthesis quality** of oligonucleotide probes that specifically recognize the sample target sequences, and their **sensitivity** for detecting low-abundance targets (Dufva 2005);
- 2) the **uniformity of the fluorescence signal intensity** over the entire spot, which mostly depends on how evenly oligonucleotide molecules are distributed among the probes (Gao et al. 2004). While it is true that the higher the molecular concentration is in a spot, the more intense the detectable signal will be (Pirrung 2002), the number of molecules that can occupy each spot is strictly limited. Due to the negatively-charged phosphate backbone of nucleic acids (Manning 1975; Gao et al. 2004), densely packed spots give rise to negative electropotentials that can repulse the target DNA sequences (Vainrub and Pettitt 2003);
- 3) the suitability of the **oligonucleotide probe surface density** for permitting the free binding of DNA strands and detection. Here, the miniaturization of the oligonucleotide spots enables better sensitivity (Ekins et al. 1990); there exists an upper density limit that may result in steric hindrance for DNA hybridization (Southern et al. 1999);
- 4) the **background signal**. A low background signal and, therefore, a well-standardized protocol for the functional treatment of the slide surface are critical;
- 5) the **reproducibility of the fluorescence signal intensities** of the probe-spots and **reproducible results between different microarrays**. Again, careful protocol optimization is critical;
- 6) the **specificity**, which is the ability to discriminate between different nucleotides (Type I error); and
- 7) the **sensitivity**, which refers to the hybridization signal/noise ratio (Type II error).

Sensitivity and specificity also depend on the oligonucleotide probe sequence (oligoprobe design) and its length, the target sequence length and concentration (PCR primer design), and the GC content of both the probe and target

sequences (Peterson et al. 2001; Religio et al. 2002; Urakawa et al. 2003; Vainrub and Pettitt 2003; Chou et al. 2004). Sensitivity is additionally defined by the hybridization step (Loy et al. 2002).



**Figure 1.** Schematic representation of the procedures involved in the development and evaluation of a microarray oligonucleotide probe set.

Figure 1 demonstrates the steps involved in the design and evaluation of a functional microarray. The design of an assay is a multilevel issue. Designing a suitable set of oligonucleotide probes may require several orders of redesign (Tonisson et al. 2002; Jaakson et al. 2003), in order to optimize cross-hybridization, specificity, binding efficiency, or similar hybridization conditions (homogeneity, uniformity).

In principle, high quality can be obtained in each aforementioned microarray technology platform by careful protocol optimization. The choice between

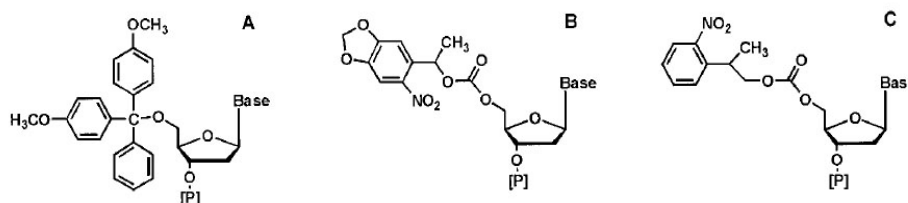
platforms therefore depends more or less on a scientist's preference. In the present doctoral thesis, we focused on oligonucleotide probe microarrays produced via maskless photocleavable *in situ* synthesis. This platform was chosen because it is a very flexible, strictly standardized, and robust protocol for producing oligonucleotide sequences in both the 3' → 5' and 5' → 3' directions (Beier and Hoheisel 1999; Stahler et al. 2006).

### I.3. Fabrication of oligonucleotide microarrays

Nucleophosphoramidite chemistry is the foundation for the synthesis of oligonucleotide probes for DNA microarrays. This chemistry was developed by optimizing oligonucleotide synthesis, and routinely achieves a > 98.5% step-wise yield in oligonucleotide probe synthesis (Caruthers et al. 1983; Beaucage 1993). Conventional oligonucleotide synthesis (3' → 5') is usually carried out on a solid support like glass, and involves the repetition of multicycles that contain four reaction steps:

- 1) **Deprotection** to release the terminal OH- group;
- 2) **Activation and coupling** of a 5'-O-4,4'-dimethoxytrityl (DMT)-protected phosphoramidite monomer;
- 3) **Capping** of the free OH<sup>-</sup> groups that failed to couple;
- 4) **Oxidation** of the internucleotide phosphite to a phosphate triester.

The deprotection (also known as de-blocking) step involves the first base, which is attached to the support and is initially inactive since the active site is blocked. The most common groups used to protect the hydroxyl group are DMT, MeNPOC, or NPPOC (Figure 2), and can be easily removed by dichloroacetic acid (DCA) (IDT 2000) or by light.



**Figure 2.** The photolabile protecting groups: **A**, DMT (acid labile protecting group); **B**, MeNPOC (photolabile protecting group, 365 nm irradiation); **C**, NPPOC [2-(2-nitrophenyl)propoxycarbonyl, 365 nm irradiation].

In the second step, synthesis of the oligonucleotide probe is performed. Base condensation with an additive monomer is activated by tetrazole, which cleaves the groups protecting the phosphorus linkage (IDT 2000). Activated base is

added to the reaction, and the active 5'-hydroxyl group of the base and the freshly activated phosphorus bind two bases together (BSI 2003). The remaining active monomers with no bound bases must next be eliminated immediately, in order to avoid false sequence alignments. Therefore, in the third (capping) step, the active 5'-hydroxyl group is inactivated with a protective group (N-methylimidazole). In the fourth step, oxidation is performed.

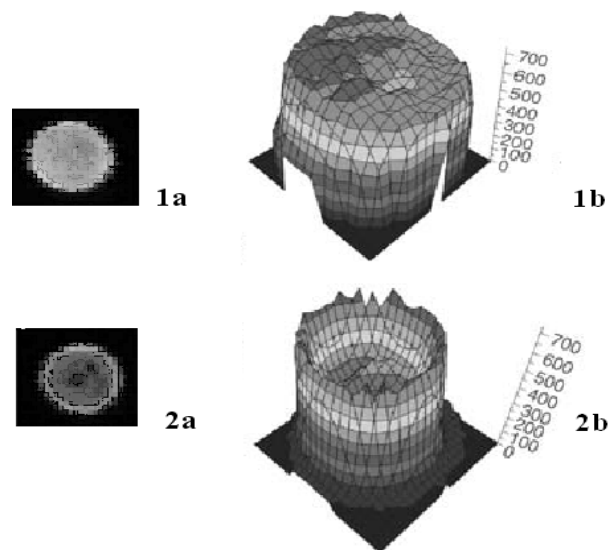
In sites where the desired base is added to the oligonucleotide probe chain, the resultant phosphite linkage is unstable. To stabilize this link, a solution of dilute iodine in water, pyridine, and tetrahydrofuran is added (IDT 2000). After the synthesis, the probes must be cleaved from the solid support and deprotected before they can be effectively used. This is done by incubating the chain in concentrated ammonia at a higher temperature. All of the protected groups are then cleaved (BSI 2003). Finally, the protecting groups of the oligonucleotide nucleobases and phosphotriesters are removed with concentrated aqueous ammonia, while at the same time the sequences are cleaved from the support.

A key step towards performing a large number of these parallel reactions in a small area ( $\sim 1 \text{ cm}^2$ ) is to control whether or not the reaction occurs at each site and in each cycle.

### **1.3.1. Fabrication of spotted oligonucleotide microarrays**

To produce oligonucleotide microarrays by spotting, pre-synthesized oligonucleotide probes (as described in chapter 1.3) are dispensed onto a chemically-derivitized glass slide surface. To efficiently attach oligoprobes to the slide, the slide surface must first be chemically modified. Glass slides are silanized with 3-aminopropyl-trimethoxysilane (Southern et al. 1992; Maskos and Southern 1993) and linked with a spacer molecule (1,4-phenylene diisothiocyanate) to increase the accessibility of the hybridization targets to their oligoprimers (Southern et al. 1999). In order to spot oligonucleotide primers onto the activated microslides, primers undergo amino modification so that they can be immobilized on the glass surface at their 5' ends.

Likewise, the fabrication of spotted microarrays is a multiparameter optimization problem, since performance quality is influenced by parameters such as the spotter type (pin, Ink-jet), pin type, humidity and temperature in the spotting unit, probe concentration, spotting buffer, immobilization chemistry, blocking technique, stringency during hybridization/washing, hybridization conditions (diffusion/mixing), probe sequence, and target preparation.



**Figure 3.** Fluorescence images of microarray spots, indicating their morphology. **1a**, Evenly distributed oligonucleotides and their 3D-view (**1b**); **2a**, Unevenly distributed oligonucleotides and their 3D-view (**2b**) (Dufva 2005).

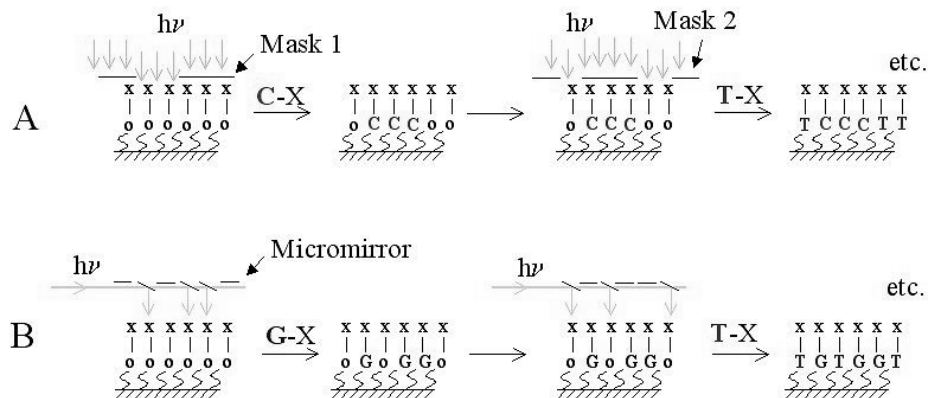
How an oligonucleotide spot performs in experiments is heavily dependent on its morphology, namely, its shape and homogeneity (Fig. 3) (Dufva 2005). Perturbations in spot structure are very common (McQuain et al. 2003) and are a significant source of signal variation (Diehl et al. 2001). The implementation of a successful genotyping microarray is heavily dependent on the slide surface, probe design, and preparation of the labelled target (Halperin et al. 2005).

Before spotting, the oligonucleotide probes are dissolved in high-salt spotting buffers (Schwille et al. 1996; Schena 1999), the pins are filled with this solution by capillary action, and the pins are transferred to their defined position on the microarray surface. The spotting buffer functions as a hygroscopic additive (Hegde et al. 2000) to avoid the too-rapid evaporation of the spotted drop from the slide, thereby maintaining a constant average spot size (Tran et al. 2002). The spotting buffer should also favour binding between the oligonucleotide probe and the functional groups on the glass surface (Hegde et al. 2000; Diehl et al. 2001; Calevro et al. 2004).

### 1.3.2. *In situ*-synthesized oligonucleotide microarrays

One of the most important advantages of *in situ* oligonucleotide synthesis relative to prespotted arrays is their greater flexibility with regards to microarray design (Singh-Gasson et al. 1999; Nuwaysir et al. 2002). Tens of thousands of oligonucleotides can be synthesized simultaneously on a microarray at relatively low cost, typically overnight. Because of this, the testing of oligonucleotides in APEX reactions and oligonucleotide microarray redesign are greatly simplified. *In situ*-synthesized oligonucleotide arrays result in more uniform attachment of the oligonucleotides at much higher densities (Beier and Hoheisel 2000).

Several methods for the *in situ* synthesis of oligonucleotide microarrays have been reported (Fodor et al. 1991; Maskos and Southern 1992; Case-Green et al. 1998; LeProust et al. 2000). Among them, mask-directed *in situ* parallel synthesis (Fig. 4A) using PLPGs (Fodor et al. 1991) has been successfully used to manufacture high-density microarrays in both the 3' → 5' and 5' → 3' directions.



**Figure 4.** Photosynthesis of oligonucleotide probes. **A**, Mask-controlled *in situ* synthesis; **B**, Maskless, micromirror-controlled, light-induced *in situ* synthesis.

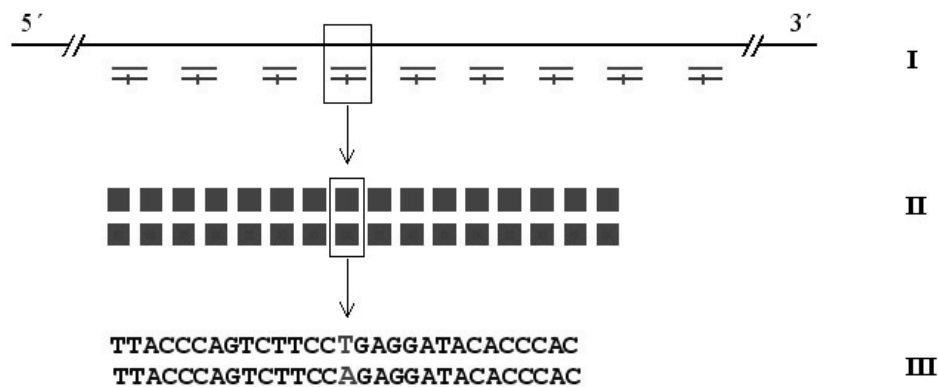
Maskless photolithographic synthesis (Fig. 4B) of oligodeoxynucleotides in the 3' → 5' direction involves the nucleophilic attack of a primary 5'-hydroxyl in the growing oligonucleotide chain by a trivalent phosphorus atom at the 3' position of the incoming phosphoramidite. In contrast, 5' → 3' synthesis requires a secondary 3'-hydroxyl to attack by a phosphorus atom located at the 5'-position of the phosphoramidite. Secondary hydroxyl groups are less efficient nucleophiles than primary hydroxyls. Therefore, chemical synthesis in the 5' →

3' direction often proceeds with a lower yield compared to the usual direction (Robles et al. 1995; Pirrung 1996; Shchepinov et al. 1997; Hudson 1998).

For 3' → 5' light-induced oligoprobe synthesis, photolabile 3'-O-NPPOC-protected phosphoramidites are used (Beier M. 2001). Typically, synthesis of the four monomeric building blocks starts from 5'-O-(MeO)<sub>2</sub>Tr-protected 2'-deoxynucleosides. For protection from exocyclic amino functions, the base-labile [4-(tert-butyl)phenoxy]carbonyl group is beneficial, since it allows mild and fast deprotection by a 1 h treatment with ammonia after synthesis (Beier M. 2001). The NPPOC moieties are introduced at the 3'-O-position by employing a mild acylating reagent generated from 2-(2-nitrophenyl)propyl carbonochloridate and 1-methyl-1H-imidazole (Beier M. 2001).

#### I.4. Hybridization-based genotyping

Allele-specific hybridization hybridizes two DNA target sequences that differ at one nucleotide position. Thus, the oligonucleotide probes must be designed so that they can discriminate between the true target and nontarget sequences that differ by a single nucleotide. The mismatch (MM) probe provides an additional background cross-hybridization control (Affymetrix Inc. 2002); the higher the specificity is, the less likely cross-hybridization and false positive generation are to occur. The hybridization of two complementary DNA (cDNA) strands is a two-step process. The first is a slow determining step, in which an initial two or three base-pairs form. This is followed by a fast “zipping-up” of the rest of the sequence (Burden 2006). In hybridization-based assays, the destabilizing effect of a single nucleotide mismatch between an oligonucleotide and its target is used to distinguish between sequence variants (Fig. 5).

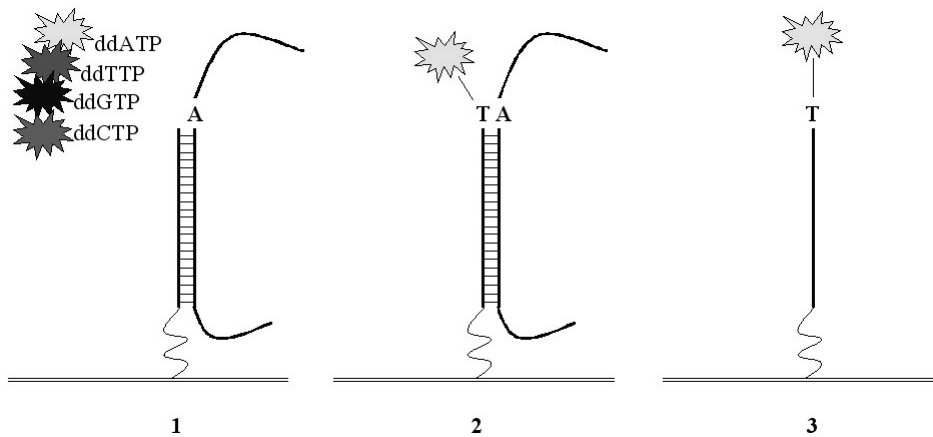


**Figure 5. I and II,** Design of a perfect match (PM) and mismatch (MM) oligonucleotide. Blue stripe represents the complementary PM sequence (usually 20–25 mere). **III,** At every PM, an additional oligoprobe different from the middle base is designed.

The stability of a hybridized probe-target complex is affected by the temperature, ionic strength around the sequences, SNP position on the target and probe sequences, and formation of secondary structures by either the probe or the target. However, optimization of the reaction conditions and primer design is necessary to perform highly multiplexed hybridization assays (Hacia et al. 1996; Hacia et al. 1998).

### I.5. APEX genotyping

The APEX reaction occurs by a two-step mechanism: targeting of DNA hybridization to complementary oligonucleotide primers, and primer extension with DNA polymerase and dideoxynucleotides (ddNTPs) that match the nucleotide at the polymorphic site (Fig. 6). Dye labelled-ddNTPs terminate the extension at the incorporation site of the base to the oligonucleotide probe (Shumaker et al. 1996; Kurg et al. 2000).

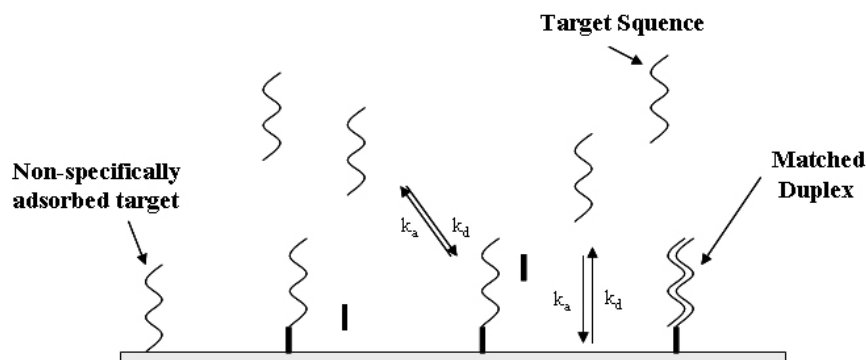


**Figure 6.** Principle of APEX. **1,** Known oligonucleotides are attached to the surface of two-dimensional arrays at their amino-modified 5' ends. **2,** The oligoprobe hybridizes the target DNA molecule and is extended enzymatically using dye terminators. **3,** Signal detection.

DNA polymerase extends the DNA strand from the 3' end; therefore, the single-stranded oligonucleotide probe must be immobilized on the microarray from the 5' end. The design of oligonucleotide probes for SNP/point mutation typing (Fig. 7A) or for insertion/deletion resequencing (Fig. 7B) is presented in Figure 7.

For unknown mutational analysis, resequencing of the gene region is suggested. Each base is identified by two unique 25-mers, one each for the sense and antisense strands, with their 3' ends one base upstream of the base to





**Figure 8.** Schematic representation of solid-phase DNA hybridization.  $k_a$  and  $k_d$  represent hybridization and denaturation of the non-specifically adsorbed targets and the reversible nonspecific adsorption and desorption of the targets to the surface.

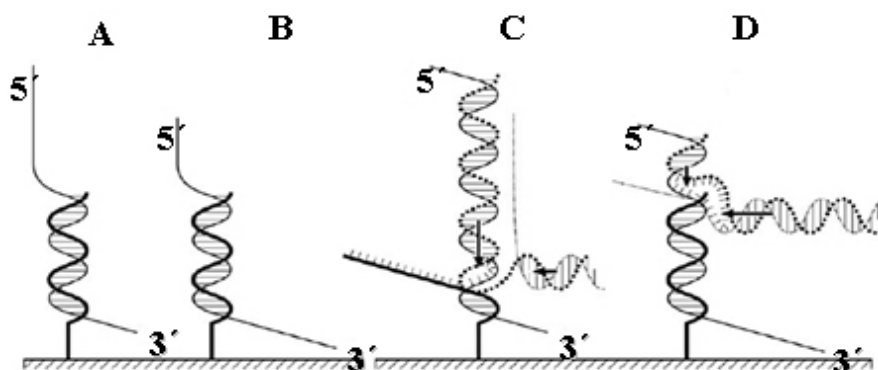
DNA hybridization is a heterogeneous reaction involving two species (Fig. 8): the target DNA in solution and the immobilized oligonucleotide probe. In oligonucleotide probe microarrays, short oligoprobes are usually chemically bound to the surface via their terminal groups. Doubled-stranded DNA (dsDNA) is a rod-like molecule, with each base-pair contributing 3.4 Å to its total length. The radius of dsDNA is 9.5 Å, and its cross-sectional area is 284 Å<sup>2</sup> (Halperin et al. 2005). In the hybridization to the oligonucleotide probe spot there are two effects: one is the steric hindrance that occurs when the probes are directly attached to the surface; the other is the reduction in the number of accessible configurations on the planar surface (Mir and Southern 1999). The distance between oligonucleotide probes can be increased by using spacer-molecules (Halperin et al. 2006) that ease the approach of free chains into the solution phase. Increasing the distance between oligonucleotide probes on the surface improves target sequence hybridization (Shchepinov et al. 1997; Riccelli et al. 2001). In addition, spacer molecules can act as moieties to introduce functional groups that modify autofluorescence or surface properties, such as charge, adhesion, or hydrophobicity / hydrophilicity (Maskos and Southern 1992; Guo et al. 1994; Schena 1999; LeProust et al. 2001; Vainrub and Pettitt 2003; Vainrub and Pettitt 2003). The oligonucleotide densities within spots typically vary between  $1.2 \times 10^{10}$ , where  $4 \times 10^{13}$  oligoprobes per cm<sup>2</sup> corresponds to  $2.5 \times 10^2 \text{ Å} \leq \Sigma_0 \leq 8.3 \times 10^5 \text{ Å}^2$  ( $\Sigma_0$  is the area per probe).

Generally, the oligonucleotide probe density controls the efficiency of target binding and the hybridization kinetics of the target-sequence and the oligonucleotide probe (Steel et al. 1998; Southern et al. 1999; Huang 2001; Peterson et al. 2001). In the lowest oligoprobe density regimes, essentially 100% of probes can be hybridized and the kinetics of binding are Langmuir-like. At higher probe densities, the efficiencies drop to ~10% and the reaction kinetics

are lower (Peterson et al. 2001). The hybridization kinetics for PM and MM DNA are distinct (Peterson 2000).

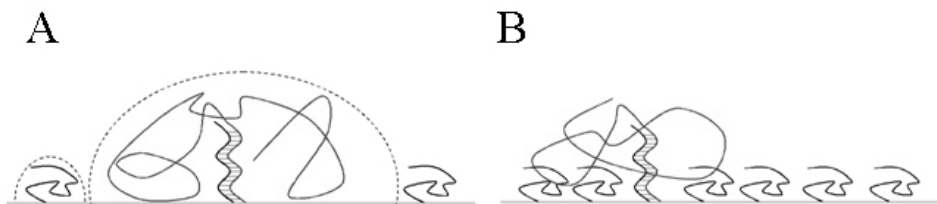
Mass transfer falls into three categories: the migration of a charged body under the influence of an electric field (i.e., a target molecule is adsorbed onto the microarray surface), diffusion, and concentration (Bishop et al. 2006). In most conventional microarrays, hybridization relies solely on diffusion (Fig. 8), and thus is a longer, diffusion rate–limited process (Borden et al. 2005). Target molecules in the solution are at a considerable distance from the oligonucleotide probes; on a molecular length scale, their diffusion to the hybridization site is most likely inefficient. The diffusion coefficient for a 250-bp DNA fragment in water at room temperature is  $\sim 2 \times 10^{-7} \text{ cm}^2/\text{s}$ , and the time constant for its diffusion along a length of 500  $\mu\text{m}$  is  $\sim 100 \text{ min}$  (Chan et al. 1995).

Several reaction rate constants for associations in solution have been measured. Using fluorescence resonance energy transfer, the hybridization rate constant between a 16-mer and its complement in solution was shown to be  $5.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ , which is four orders of magnitude smaller than the estimated diffusion-limited rate of  $8.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$  (Parkhurst and Parkhurst 1995). Intramolecular base pairing can be involved in the formation of secondary structures (such as hairpin loops) that clearly affect hybridized complex stability (Du et al. 2003; Du et al. 2005; Jung and Van Orden 2006) and hybridization reaction rates. The effect of intramolecular base pairing can be minimized to achieve better detection sensitivity by increasing the hybridization temperature or by designing shorter oligoprobes.



**Figure 9.** Interactions between an immobilized oligoprobe and its target PCR product strand. The target strand is hybridized to the oligoprobe (A) leaving a long and a short 5' overhang of the product strand (B); (C) a free complementary strand of the target product hybridizes to the overhanging tail, generating a secondary complex that destabilizes the oligo-hybridized complex; (D) the free hybridized region of the target sequence generates a hybridized complex (Peytavi et al. 2005).

In oligoprobe hybridization experiments, the targets that hybridize to their complements on the microarray surface are usually significantly longer than the oligonucleotide probes (Fig. 9). Because of this, the tails do not overlap and the target sequences form duplexes with free complementary sequences in the solution (Fig. 9, C and D). These duplexes begin to concur with probe/DNA duplexes in term of the concentration of detectable material, reducing the equilibrium constant of hybridization. It also becomes necessary to allow for crowding of these unhybridized “tails” as the fraction of hybridized probes grows (Fig. 10) (Halperin et al. 2005).



**Figure 10.** **A, B** Oligoprobe density within a spot-feature; **B**, The overhanging target sequence tail influences the further hybridization of the next sequence on the oligoprobe.

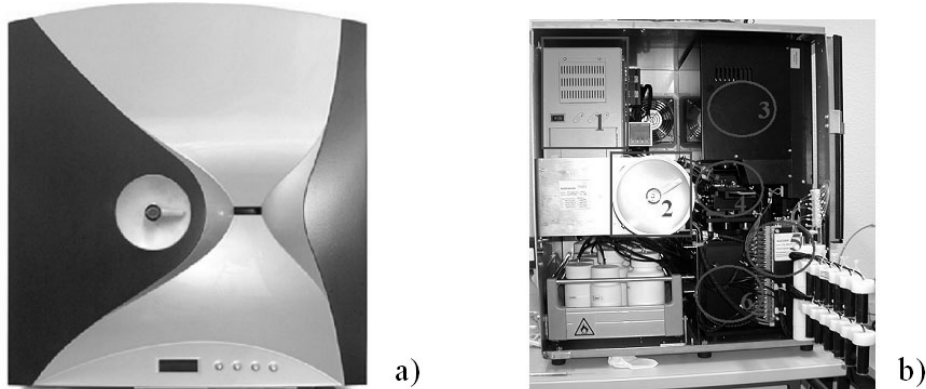
Slow diffusion of the target molecules can limit the overall hybridization rate (Borden et al. 2005). Furthermore, steric hindrance between a surface probe and its target molecules (Fig. 10) (Peterson et al. 2001) also decreases the overall hybridization rate (Gadgil et al. 2004). Whether the limiting step is reaction- or diffusion-limited depends entirely on the total time of the hybridization reaction (Pappaert 2003). Short time-scales are characterized by reaction-limited hybridization, given the readily available probe at the initial bulk concentration. After this initial time-period, probe depletion renders the process diffusion-limited.

Hybridization conditions that must be optimized include the composition of the solution, temperature (Blasko 1996), time, base composition and length of the target probe (Chan et al. 1995; Stillman and Tonkinson 2001), target concentration and probe density (Chan 1997), association/dissociation kinetics (Blasko 1996; Jensen et al. 1997), method of probe immobilization (Livshits 1996; Yang 1998), mass transfer (Myszka 1998; Mason 1999), and the relative ratio of solute diffusion to the intrinsic reaction rate (Chan et al. 1995). Typical hybridization temperatures vary from  $30^{\circ}\text{C} \leq T \leq 60^{\circ}\text{C}$ , depending on the oligoprobe length and GC content (Maskos and Southern 1993, Pirrung 2000). Micromixing can accelerate the hybridization kinetics and improve hybridization uniformity (Liu et al. 2003). Various micromixing methods have been developed, including DNA migration enhancement by a direct electric field (Edman et al. 1997; Sosnowski et al. 1997), dynamic DNA hybridization using paramagnetic beads (Fan et al. 1999), and the use of a microporous three-

dimensional biochip through which the hybridization solution is pumped continuously (Cheek et al. 2001).

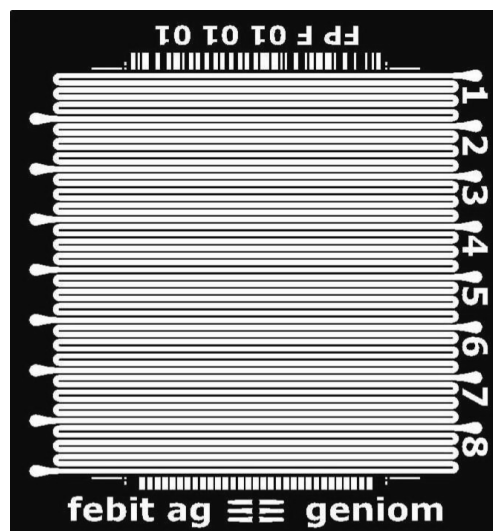
## 1.7. Geniom One technology

Geniom One (Fig. 10) contains *in situ* DNA synthesis, reaction, and detection units within one instrument, allowing oligonucleotide synthesis, reactions (hybridization, APEX), washing, and signal detection to be carried out in the same microchannels.



**Figure 11.** (a) The Geniom One system. (b) 1, Embedded PC, the internal computing facility; 2, DNA synthesizer module; 3, High resolution optics for light projection and detection; 4, Central processing unit takes up the biochip cartridge and aligns it to Geniom One optics and fluidics; 5 and 6, DNA synthesizer modules.

The three-dimensional microchannel cartridge (Fig. 11) is divided into eight individual subarrays, each consisting of 6,776 features that are independently available for different assays (Beier and Hoheisel 2002). Its current version can synthesize arrays of up to 48,000 oligoprobes (Fig. 12). Each analysis step – *in situ* synthesis of the oligonucleotide microarray, hybridization of the PCR products, and detection of signal intensities – is performed within the same device. Controlled by a mask-free, light-controlled process, oligonucleotide probes can be synthesized in either a 3' → 5' or 5' → 3' direction. The entire set of oligonucleotide probes is synthesized simultaneously directly onto the microarray surface inside the biochip microchannels. The microarray oligonucleotide matrix can therefore be analyzed once, and afterwards the researcher can decide which oligoprobes need to be redesigned.



**Figure 12.** Biochip of 8 separate microchannels with individual fluid control. Each microchannel serves as a reaction carrier for *in situ* synthesis and as a hybridization chamber, and contains 15,624 individual features. Each array may be loaded with a different sample or combined to obtain 124,992 features for high-density applications.

Using a high-pressure mercury discharge lamp of 350–750 nm, the digital micromirror device (Fig. 11b) performs light-activated spatially resolved deprotection of the photolabile groups immobilized inside the biochip microchannels. Each microchannel contains 6000 individual features, and each array may be loaded with a different sample or combined to obtain 48,000 total features. The deflection of one of 786,432 individually addressable micromirrors [Digital micromirror device (DMD), Texas Instruments] is done by software control. The size of the oligonucleotide feature is  $34 \times 34 \mu\text{m}$ . Within the channel, there are three oligonucleotide probes per row and 180 probes per column. When amidite building blocks are added, coupling takes place at the previously deprotected spots. Repeated cycles of photo-deprotection and amidite coupling result in specific oligonucleotide probe sequences.

A microfluidics interface connects the biochip to the reservoirs containing synthesis chemicals (Fig. 10b). The microfluidics system is kept in an inert argon atmosphere. Geniom One detects fluorescence via digital imaging, with an 8 megapixel CCD camera that uses a high-aperture macro lens and records images of the fluorescence pattern on the biochip surface. A background image is subtracted from the actual sample recording, the image is saved as a standard TIFF file, and Geniom One calculates the signal intensities from the image data and allocates these to the corresponding features. Results are saved as standard txt files and may be exported as such or as Excel data.

## **2. PRESENT INVESTIGATION AND DISCUSSION**

### **2.1. Aims of the present study**

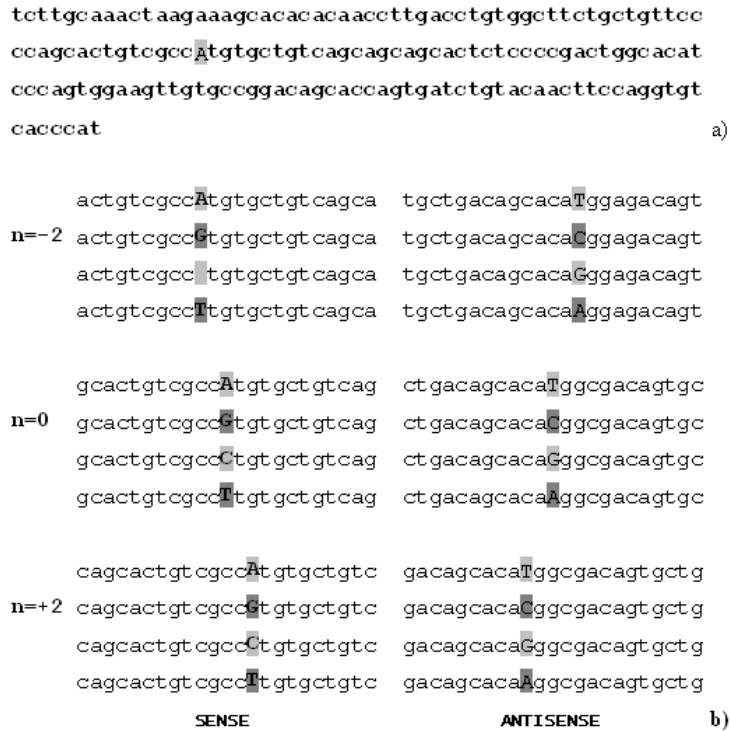
The aim of this study was to establish an arrayed primer extension (APEX) reaction on an *in situ*-synthesized oligonucleotide microarray in both the conventional 5' → 3' and unconventional 5' → 3' directions using the Geniom One platform, and to test the usefulness of the Geniom One platform for designing oligonucleotide arrays for large-scale diagnostics applications.

### **2.2. Genotyping by hybridization**

#### **2.2.1. Immunochip development (References I and II)**

The oligonucleotide microarray for genotyping by hybridization was designed to detect 99 SNPs in 68 genes that have putative functional significances in the occurrence of hay-fever. The experimental parameters of the SNP position in the oligonucleotide, overall probe lengths, and hybridization temperature and duration were analyzed and optimized. For each SNP, all four possible sequence variations were applied to the microarray (Fig. 13). The PM/MM approach used for hybridization was the principal reason for differences in signal intensities. Mismatch probes immediately provided information on the background signal level.

Positional effects were tested by moving the polymorphic nucleotide from the centre to positions +2 and -2 or to positions +1 and -1 of the oligonucleotide probe-feature (Fig. 13). This shift resulted in differences in signal intensities, but did not add to the overall amount of information that could be gathered from an experiment. Consequently, we decided to use only probes that contained the respective SNP in a central position, but placed three copies at different locations in the microarray.

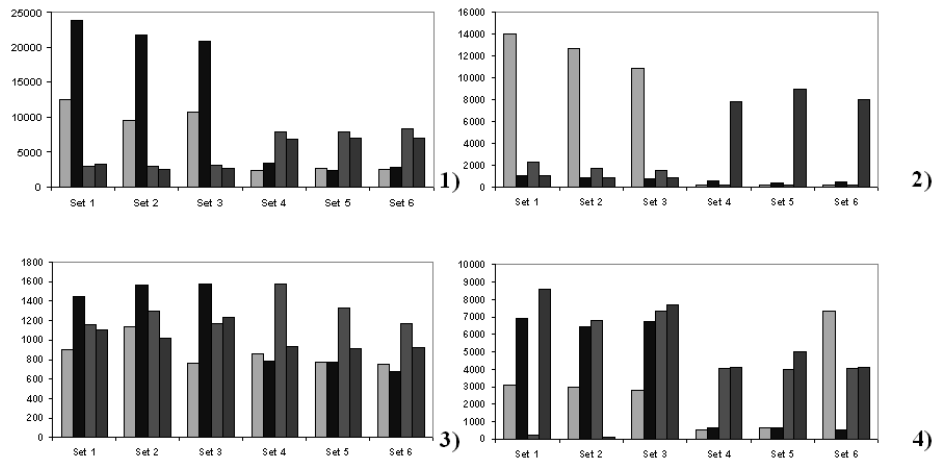


**Figure 13.** Design of 23-mer oligonucleotide probes for SNP detection. **(a)** PCR product of 166 bp. **(b)** The set of oligonucleotides (12 for sense and 12 for antisense strands; at  $n = 0$  the allele is located in the middle of the oligonucleotide probe; at  $n = -2$  and  $n = +2$ , the SNP is shifted by 2 nucleotides to the left and right, respectively).

Several oligonucleotide probe lengths were also tested, using an array with 19-, 21-, 23-, 25-, and 27-mer oligonucleotides (Fig. 14). While longer sequences usually produce higher signal intensities, shorter oligonucleotides permit better discrimination of single base differences due to the more destabilizing effect of a mismatch. Ideally, the detected intensity for PM oligonucleotide probes differs from MM probes by a minimum of 30-fold. In hybridization, the signal intensity depends directly on the sequence GC content of the probe. We observed that the signal intensities of both the fully matched ( $I_1$ ) and mismatched probes ( $I_2$ ) increased with length, while the discrimination ( $I_1/I_2$ ) improved as the oligonucleotide length decreased (Fig. 14).

	27	mean $I_1/I_2$	25	mean $I_1/I_2$	23	mean $I_1/I_2$	21	mean $I_1/I_2$	19	mean $I_1/I_2$
0	25577		18249		15081		9505		4841	
	<b>47017</b>	<b>1.6</b>	<b>38201</b>	<b>1.7</b>	<b>32529</b>	<b>2.0</b>	<b>24681</b>	<b>2.5</b>	<b>25881</b>	<b>5.3</b>
	28201		20809		15889		8681		3009	
1	29361		23113		16297		9881		3641	
	28057		22745		13017		9089		8105	
	<b>45673</b>	<b>1.6</b>	<b>38801</b>	<b>1.7</b>	<b>27609</b>	<b>2.1</b>	<b>22921</b>	<b>2.5</b>	<b>29017</b>	<b>3.6</b>
-1	27001		21449		11769		6697		4777	
	28641		20561		11449		7577		5601	
	24585		20313		16041		15833		8233	
	<b>43497</b>	<b>1.7</b>	<b>39081</b>	<b>1.8</b>	<b>35417</b>	<b>1.8</b>	<b>31705</b>	<b>2.0</b>	<b>29625</b>	<b>3.6</b>
	25745		21177		17913		14489		5961	
	25033		20857		17289		14761		6537	

**Figure 14.** The dependence of the signal intensity on oligonucleotide length. Hybridization was performed at 45°C.  $I_1/I_2$ , ratio of signals at the PM and MM oligonucleotides. 27, 25, 23, 21, and 19, oligomer lengths. The SNP was located either at the centre of the oligonucleotides (0) or shifted by two bases in either direction (+1, -1).



**Figure 15.** Differences in the oligonucleotide performance. Sets 1–6, oligonucleotide probes designed for detecting SNPs. Columns indicate the signal intensity of oligomers that represent (left to right) the A, G, C, or T variant of a sequence. Sets 1–3, data produced at oligonucleotide replicate microarray positions that represent the oligoprobe strand; Sets 4–6, signal intensities produced by the cDNA strand. 1) Heterozygous sample, 2) Homozygous sample, 3) Oligoprobe was predicted *in silico* to perform well but failed in the experiment, 4) Oligomer with a high degree of cross-hybridization.

The 99 PCR products were pooled prior to labelling and were hybridized concomitantly (Fig. 15). For each sample, analysis was repeated up to four times.



To assess the genotyping accuracy, ten PCR products of heterozygote calls obtained from the microarray analyses were subjected to gel-based DNA sequencing for confirmation. In all cases, the results were in full agreement.

While the *in silico* process eliminated 9% of the initial 141 SNPs, which had been picked purely on the basis of their potential association with the occurrence of hay-fever, the subsequent empirical validation excluded another 20% of these oligoprobes. This illustrates the importance of validating microarray design in actual experiments. Even in analyses based on the continuous detection of the hybridization and dissociation process (dynamic allele-specific hybridization) (Jobs et al. 2003) the selection is critical, although the analysis of duplex association and dissociation curves permits more discriminative and accurate SNP detection. However, the specificity and stability of DNA duplex formation depends strongly on sequence and base composition (Wetmur and Davidson 1968; Breslauer et al. 1986). Also, the target sequence on either side of the SNP position plays an important role, since secondary structures may strongly effect the hybridization behaviour of a sample (Southern et al. 1999). Therefore, it is frequently insufficient to predict hybridization performance merely on the basis of theoretical calculations. The use of flexible *in situ*-synthesized oligonucleotide microarrays appears to be an efficient and attractive method for fast and cost-efficient pre-screening of candidate SNPs for eventual high-throughput genotyping.

### **2.2.2. Simultaneous typing of 81 human papillomaviruses (Reference II)**

Human papillomavirus (HPV) infections are associated with ~ 15% of cancer cases (zur Hausen 1991), and their prevalence is very common (Bosch et al. 2001). Large numbers of new types of HPV have been detected, and the complete sequences of 118 distinct HPV types have been described to date (de Villiers et al. 2004). HPVs are quantitatively the most important group of viruses associated with benign and malignant neoplasias in humans (Lowy et al. 1994). They are commonly grouped into high- and low-risk categories according to their known epidemiological associations (Munoz et al. 2003). Infection with multiple HPV types occurs frequently, and type-specific HPV DNA detection in patient samples is of utmost importance in clinical management.

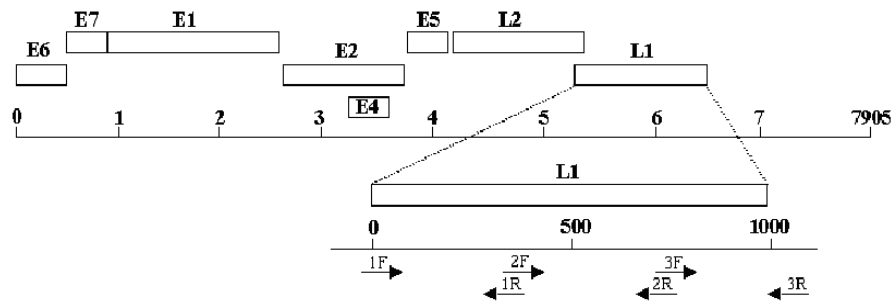
The papillomavirus is a small icosahedral DNA virus containing a double-stranded, circular DNA genome of ~ 8 kb. HPV genomes comprise seven early (E1-E7) and two late (L1 and L2) open reading frames (ORFs). The HPV sequences included in this study and their accession number were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>):

HPV1 (U06714), HPV2 (X55964), HPV3 (X74462), HPV4 (X70827), HPV5 (M22961), HPV6 (AF092932), HPV7 (X74463), HPV8 (M12737),

HPV9 (X74464), HPV10 (X74465), HPV11 (M14119), HPV12 (X74466), HPV13, (X62843), HPV14 (X74467), HPV15 (X74468), HPV16 (K02718), HPV17 (X74469), HPV18 (X05015), HPV19 (X74470), HPV20 (U31778), HPV21 (U31779), HPV22 (U31780), HPV23 (U31781), HPV24 (U31782), HPV25 (X74471), HPV26 (X74472), HPV27 (X74473), HPV28 (U31783), HPV29 (U31784), HPV30 (X74474), HPV31 (J04353), HPV31 (J04353), HPV32 (X74475), HPV33 (A07020), HPV34 (X74476), HPV35 (M74117), HPV36 (U31785), HPV37 (U31786), HPV38 (U31787), HPV39 (M62849), HPV40 (X74478), HPV41 (X56147), HPV42 (M73236), HPV44 (U31788), HPV45 (X74479), HPV47 (M32305), HPV48 (U31789), HPV49 (X74480), HPV50 (U31790), HPV51 (M62877), HPV52 (X74481), HPV53 (X74482), HPV54 (U37488), HPV55 (U31791), HPV56 (X74483), HPV57 (U37537), HPV58 (D90400), HPV59 (X77858), HPV60 (U31792), HPV61 (U31793), HPV63 (X70828), HPV65 (X70829), HPV66 (U31794), HPV67 (D21208), HPV69 (AB027020), HPV70 (U21941), HPV71 (AB040456), HPV72 (X94164), HPV73 (X94165), HPV74 (AF36130), HPV75 (Y15173), HPV76 (Y15174), HPV77 (Y15175), HPV80 (Y15176), HPV82 (AB027021), HPV83 (AF151983), HPV84 (AF293960), HPV85 (AF131950), HPV86 AF349909), HPV87 (AJ400628), HPV89 (AF436128), HPV90 (AY057438), HPV91 (AF419318), HPV92 (AF531420).

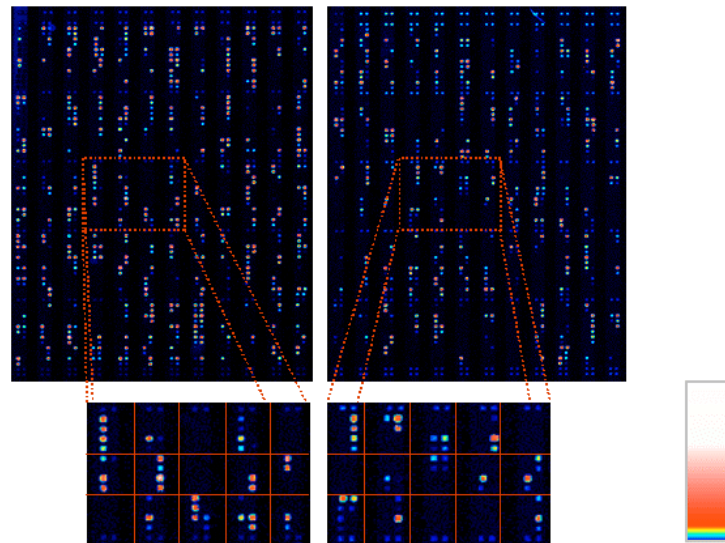
#### 2.2.2.1. Simultaneous typing of 81 HPVs by hybridization on an oligonucleotide microarray

Probes specific for regions of relatively low homology (L1 ORF) (Fig. 17) were designed for each HPV type. The L1 region was chosen because this region of PV genomes is the most variable. Both sense and antisense strands were used in the hybridization experiments. To predict cross-reaction events, all calculated oligonucleotide probe sequences were checked *in silico*. To check the effect of probe-length, 18-, 20- and 22-nt oligonucleotides were synthesised. For every oligonucleotide probe length, 16 sense and 16 antisense oligonucleotides were created, yielding 96 oligomers per HPV type. Type-specific primers for the PCR amplification of the L1 regions of 81 HPVs were designed. To amplify the entire L1 region, three amplification reactions were performed (Fig. 17). The PCR product size varied between 150 and 700 bp.

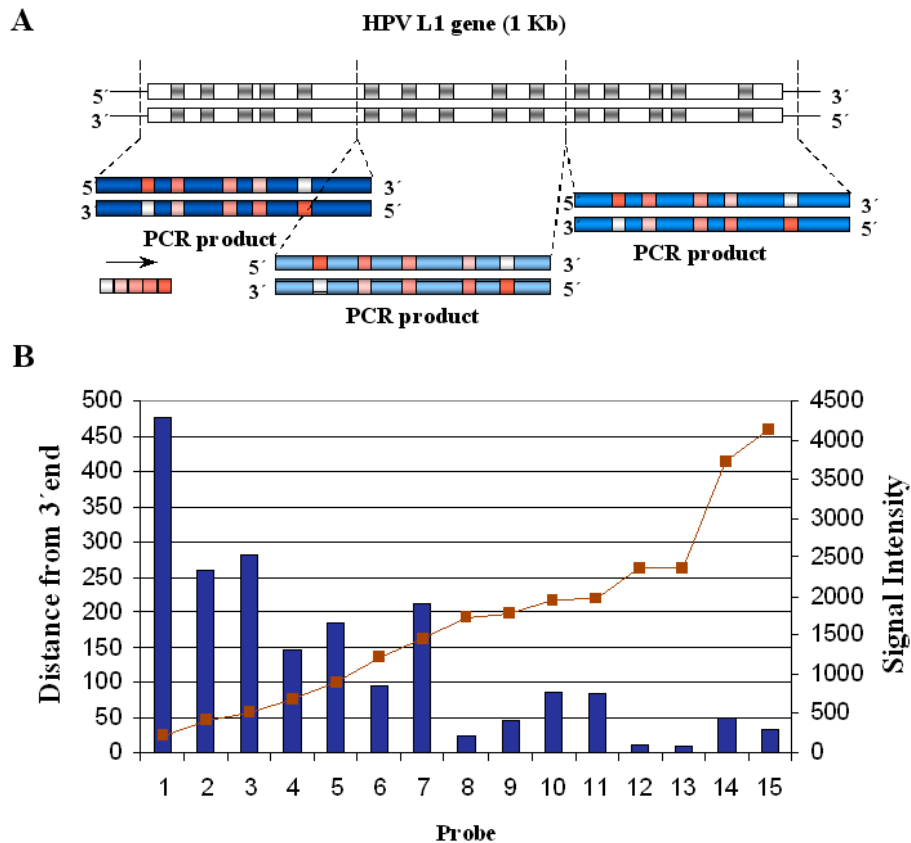


**Figure 17.** HPV genome alignment and primer design for amplifying the L1 gene. Proteins E1 and E2 are the two viral proteins required for viral DNA replication; E4 and E5 are needed for amplification of the viral genome in the upper layers of the epithelium; E6 and E7 cooperate to immortalize cells and also induce genomic instability; and L1 and L2 form the viral capsid. F and R are the forward and reverse PCR primers, respectively.

Oligonucleotide probes for 14 HPV types (2, 3, 4, 6, 11, 16, 18, 38, 53, 63, 74, 75, 76, and 80) and their related MM probes were synthesized. Thus for each HPV type, 192 oligonucleotide probes were synthesized *in situ* on the microarray. Hybridization reactions were first performed with two pools of the 14 HPV types. In addition, oligonucleotide probes specific to HPV types 74 and 76 were synthesized in order to have additional negative controls.



**Figure 18.** Performance of selected oligonucleotide probes for detecting different HPV types. Hybridization was performed at 45°C for 3 h. White-to-blue scale represents the signal intensity.



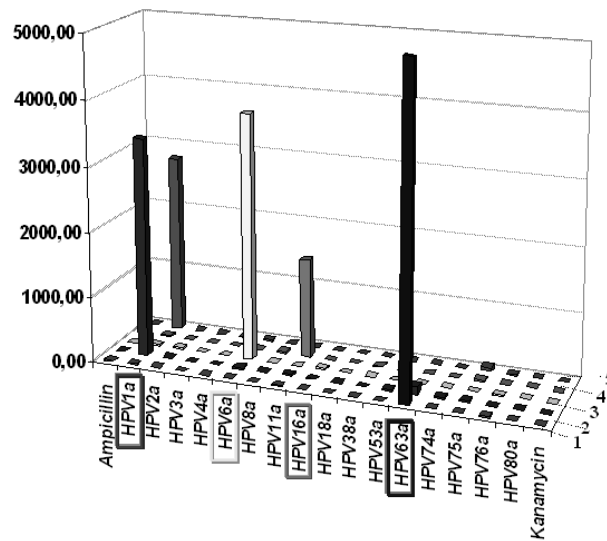
**Figure 19.** A, Alignment of HPV type-specific oligoprobe target sequences on HPV DNA PCR amplicons. The overlay of white to dark red represents the theoretical signal intensity spectrum from most to least intense; B, The presentation of measured signal intensities (blue columns) and the distance of the oligonucleotide probe from the 3' end of the PCR product (red square).

Due to steric hindrance, the binding capacity of a target DNA to its immobilized oligoprobe and the hybridization efficiency are highly dependent on the position at which the target nucleic acid binds to the captured probe (Peytavi et al. 2005). We found that the detectable fluorescence signal depended on the position of the amplicon target sequence (Fig. 19). The closer the target sequence was situated to the 3' end of the PCR product, the higher the intensity of the measured signal was. The weakest signal was obtained at the 5' end. Thus, the successful performance of a specific oligoprobe is directly related to the PCR primer design.

To optimize the specific hybridisation conditions, the influence of several parameters (e.g., temperature, time) was evaluated. Hybridisation results were

interpreted by calculating the signal intensities. As expected, the amount of cross-hybridisation decreased with increasing temperature, thereby improving the specificity. Oligonucleotide sequences and appropriate reaction conditions were selected, and from the 96 probes per HPV type, 15 remained that were used to generate the next array.

In diagnosing an HPV infection, it is vital to know how the HPV types are distributed in HSIL and whether a particular type is representative of those that cause cancer. The preliminary data obtained with the designed HPV-typing oligonucleotide microarray displayed a high sensitivity and specific detection rate for samples with multiple HPV types. Ideally, a large number of HPV type-specific oligonucleotides could be easily spotted on the microarray (Fig. 18).



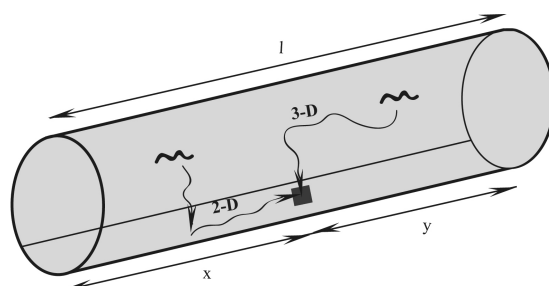
**Figure 20.** Hybridization of single HPV types on the *in situ*-synthesized microarray. 1, 2, 3, 4, and 5 are different experiments. Ampicillin and kanamycin are the control oligonucleotide probes for background settings in order to quantify the detected fluorescence signal intensities.

The performance of the current oligo array platform is defined by its ability to discriminate between multiple HPV types. In the case of multiple infections, multiple hybridization signals were detectable, and single HPV types could be specifically detected (Fig. 20). Thus, the selected subset of oligonucleotide probes synthesized *in situ* by Geniom One in the conventional 5' → 3' direction permitted the successful detection of 81 HPV types in parallel.

### 2.3. Mutational analysis and SNP typing with APEX (References III and IV)

Typically, 10–30% of oligonucleotide primers need to be redesigned to convert all sites (mutations / SNPs) into functional assays (Tonisson et al. 2002; Jaakson et al. 2003). This process is time-consuming, since several orders of magnitude of new oligonucleotide primers are required, and costly, since some oligonucleotides will only be used a few times. With SNPs, this is not a major issue because there are a large number to choose from without loss of genetic information. However, with defined mutations the situation is more complex, and new primers are usually needed, each with modified properties or further modifications.

Integrating APEX, which is optimized for prespotted oligonucleotide arrays on microscope slides, into a Geniom One microchannel biochip is, in principle, a complex multi-parameter optimization problem. The target sequences strongly depend on mass transport, which slows hybridization, and the microchip microchannels strongly affect the success of target binding to the oligoprobes due to mass transport constraints (Fig. 21).

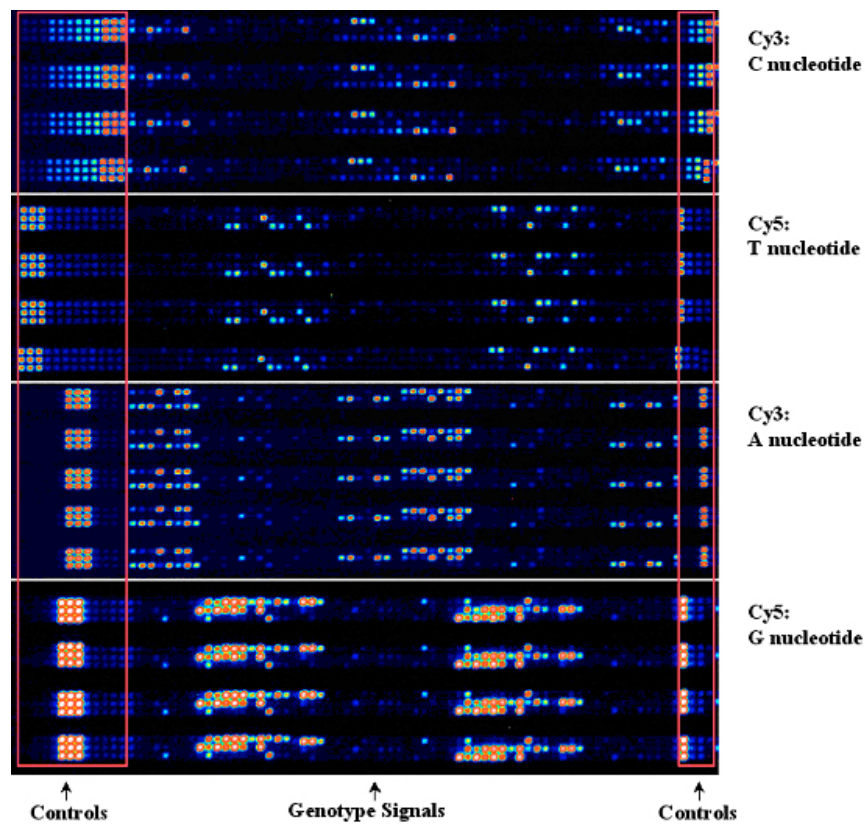


**Figure 21.** Schematic of target binding to oligonucleotides within the microchannels. 3-D, binding by direct diffusion from the bulk solution; 2-D, binding by a two-step reaction mechanism, adsorption on the surface followed by a two-dimensional search for binding sites along the surface;  $x$  and  $y$ , dimensionless coordinates of an oligonucleotide feature within the microchannel; and  $l$ , length of the microchannel in cm.

To demonstrate how the biochip microchannel geometry influences hybridization reaction kinetics, we applied a two-compartment model (TCM) (Schuck and Minton 1996; Schuck and Minton 1996; Goldstein et al. 1999). The TCM is a mathematical tool used to analyse mass transport-dependent reactions (Schuck and Minton 1996; Schuck and Minton 1996; Goldstein et al. 1999). It incorporates mass transport parameters that depend on oligonucleotide binding: mass transport from the bulk to the reaction compartment and subsequent binding. The main advantage of TCM is its ability to quantitatively analyse a particular system without requiring complicated prediction parameters, such as the binding site density, diffusion constant, stirring velocity, or reaction mecha-

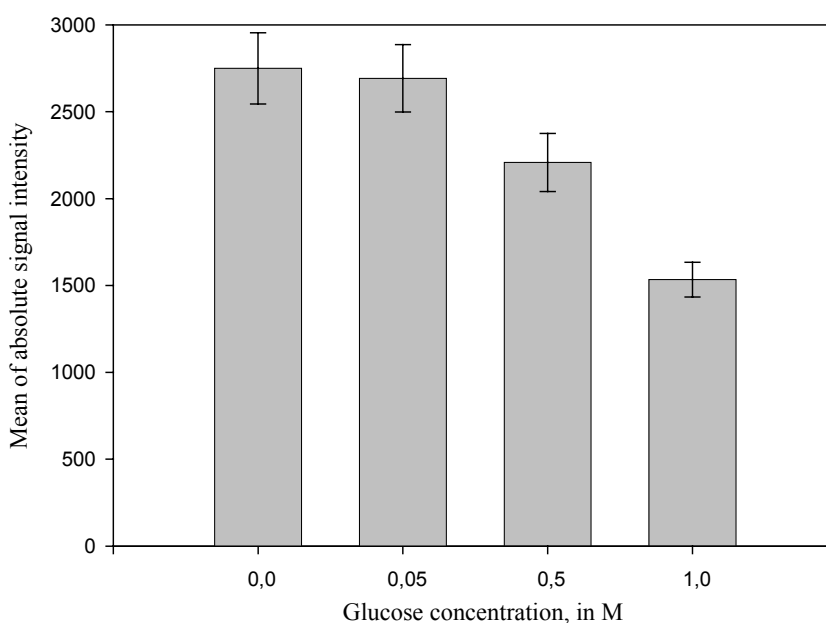
nisms. In addition, TCM can simulate a wide range experimental conditions that are difficult or even impossible to analyse in a practical context.

A set of 6,776 oligonucleotide probes, including replicates and controls, that exhibited similar hybridization and enzymatic single base extension characteristics under standard conditions were synthesized. For the APEX reactions, 25-mer sense and antisense PM oligoprobes were synthesized in the 5' → 3' direction *in situ* within the microchannels. Mutational analysis was performed by a single base extension, complementary to the template and with dye-terminators, using DNA polymerase (Thermo Sequenase). This DNA polymerase specifically extends the 3'-end of the primer with a labelled ddNTP analog that is complementary to the nucleotide at the variable site in patient DNA (Tonisson et al. 2002). The APEX signals detected after the extension reaction are presented in Figure 22.



**Figure 22.** Images obtained by scanning a “subarray” at two wavelengths, using Cy-3 and Cy-5. Results from one individual genotyped by APEX are shown for a panel of 450 SNPs/mutations, with oligonucleotide primers for both DNA strands. The obtained signals are reproduced with an artificial rainbow scale, with blue for low signals to white for saturated signals.

The slowdown of hybridization reaction rates due to mass transport effects can strongly impact many parameters, as well as the overall performance of the reaction (Kusnezow et al. 2006). In principle, the viscosity is inversely proportional to the diffusion coefficient according to the Stokes-Einstein relationship, and thus to the rate of a mass transport-limited reaction. To examine the potential effect of mass transfer in the biochip microchannels, the viscosity of the solution was varied by adding different concentrations of glucose [0 (reference), 0.01, 0.1, and 1 M]. Low glucose concentrations had little or no effect on signal intensities (Fig. 23), but the overall mean signal intensity at 1 M glucose was nearly half of that in the solution without glucose (56%). Because 1 M glucose increased the viscosity of the water solution by a factor of two (Toda and Furuse 2006), this experiment clearly demonstrates the presence of diffusional constraints in the reaction solution.



**Figure 23.** Dependence of the 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.

To determine the reproducibility of the APEX signals, different oligonucleotide probes were synthesized in 11–12 replicates per tested condition. These were analyzed at different time points from 10 min to 12 h of incubation, with various concentrations of the corresponding PCR products (0.1, 0.5, 1, 2, and 10 ng/ $\mu$ L). The obtained signal intensities were analyzed as suggested previously (Kusnezow et al. 2007) to estimate the overall effects which influence

the reaction in the microarrays. Specifically, individual signal intensities were normalized by the mean of all signals at the corresponding time points. The concentrations and CV% values from more than 40 normalized values were calculated over all tested oligonucleotide probes. Additionally, 48 positive controls for each tested condition were analyzed to obtain values for the experimental variation in the system.

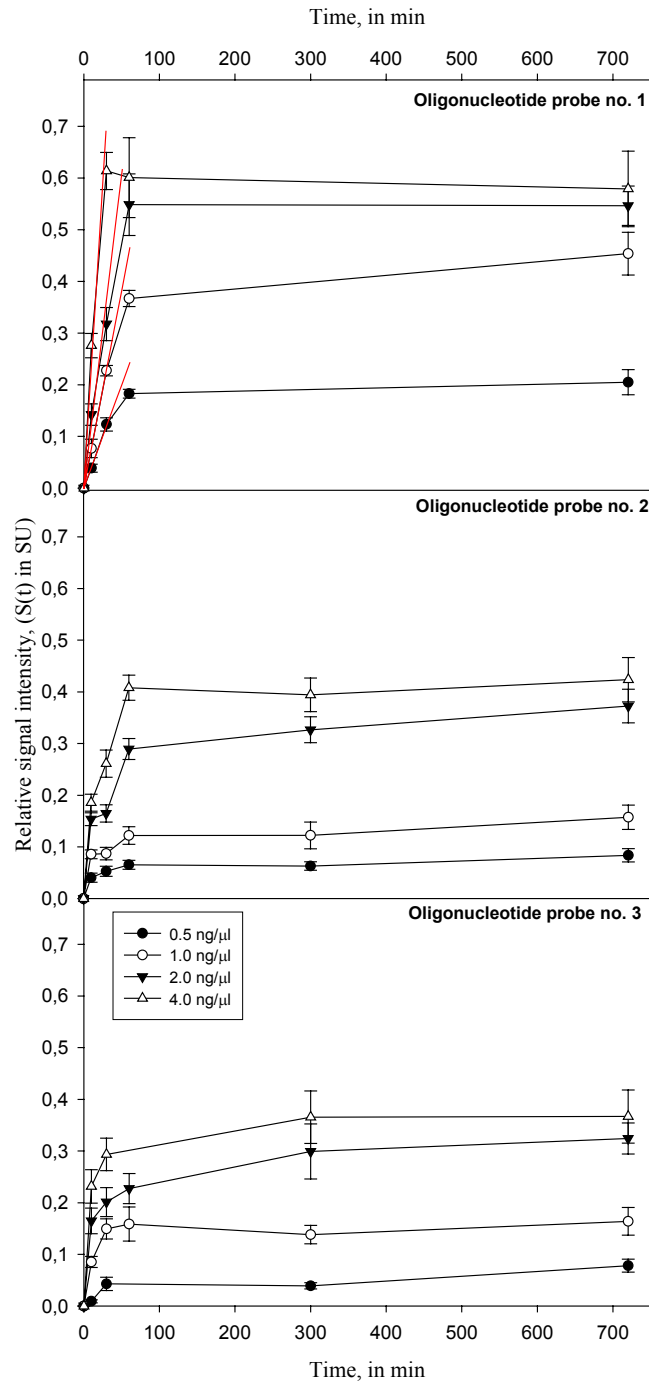
The observed variance in signal intensities in general was similar for all sequences. The highest variance was observed at lower concentrations (0.1, 0.5, and 1 ng/ $\mu$ L) and shorter incubation times (up to 1 h), while higher target concentrations (0.1 to 10 ng/ $\mu$ L) had 2–3-fold reduced CV% values. In the case of low concentration, increased incubation time led to a slight but significant improvement in CV%.

The PCR product concentration, incubation time, and the attainment of thermodynamic equilibrium by the optimal choice of both parameters are crucial factors for improving the signal-to-noise ratio and reproducibility of an APEX reaction. To optimize these parameters, progression binding curves for 0.5, 1.0, 2.0, and 4.0 ng/ $\mu$ L amplicon concentrations were analyzed at incubation times of 10, 30, 60, 300, and 720 min (Fig. 24).

Ten replicates were synthesized per oligonucleotide probe. To minimize the influence of competitive binding from neighbouring probes, replicas within the same channel were positioned at the maximal distance from each other. For each oligonucleotide probe, the signal intensities ( $S(t)$ ) were normalized by the maximally attainable signal, so that  $S_{max}$  was always equal to 1 and  $S(t)$  varied from 0 to 1. The characteristic time of exponential signal development could be theoretically attained between 20–50 min (Fig. 24), while nearly saturated signals needed up to 2.5 hours.

Additionally, by converting the target concentrations to their molar forms by using the known lengths of the PCR products, equilibrium dissociation constants ( $K_d$ ) for the analyzed interaction pairs could be estimated. The reaction temperature in APEX is high in order to ensure enzymatic processing specificity, and high temperatures reportedly increase the  $K_d$  of solid phase hybridizations (Okahata et al. 1998). This consequently leads to lower signal intensities. Therefore, the optimal hybridization temperature in an APEX reaction is a compromise between the optimal enzymatic activity and the maximally attainable signal intensity.

The values obtained in this study were consistent with each other. Despite the relatively low interaction affinities (within the mid-nanomolar range), these values indicated that all analyzed interactions were slowed by the same mass transport effect. The incubation conditions (temperature, time) were optimized, and an entire set of oligoprobes (for both sense and antisense strands) was synthesized for 450 SNPs and mutations, resulting in 900 probes in total. These were synthesized *in situ* in the 5'  $\rightarrow$  3' direction in four replicates.



**Figure 24.** 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.

To validate the assay, four genomic DNA samples were tested. A blind experiment was then performed with 36 anonymous individuals to determine the assay quality. The results were compared with those obtained by Asper Biotech from a prespotted oligonucleotide microarray. All found mutations were confirmed, and the results of four of these individuals completely coincided with those obtained by Asper Biotech. For the remaining 32 individuals, the SNP call rate for the sense and antisense strands were 96% and 87%, respectively, with respect to all variations. The total concordance of the detection results (for mutations and SNPs together), estimated over 36 individuals, was 98% compared to the reference data from Asper Biotech.

## CONCLUSIONS

1. The flexible, maskless photolithographic synthesis of microarrays on a Geniom One platform was used to produce oligonucleotide arrays in the  $3' \rightarrow 5'$  and  $5' \rightarrow 3'$  directions. Reaction parameters were determined for diagnostic purposes for two different diagnostic assay formats: oligonucleotide hybridization and APEX. The main aim was to understand how the biochip microchannel, as the reaction vessel, influences the hybridization or APEX reaction parameters. Parameters affecting the success of the APEX reaction, such as hybridization temperature, PCR product concentration, incubation time, and signal intensity variability, were analyzed and optimized.
2. Three distinct diagnostic microarrays were established and evaluated: SNP genotyping of candidate genes associated with hay-fever, genotyping of 81 HPV types for differential diagnostic purposes, and mutational analysis of the ABCR gene important in Stargardt disease. The first two tests were based on target hybridization to a  $3' \rightarrow 5'$  oligonucleotide array. The third test was developed for the APEX method and used  $5' \rightarrow 3'$  oligonucleotide arrays.
3. The maskless photolithographic production of microarrays *in situ* on a bench top instrument was shown to be a new, reliable, fast, flexible, and economic way to develop diagnostic assays for microarrays, which can then be produced and used on a large scale using the traditional slide format.

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## SUMMARY IN ESTONIAN

### Eestikeelne pealkiri

Oligonukleotiidide mikrokiibi tehnoloogia platvormi on senini käsitletud kui kiiret, odavat ja paindlikku genoomse variatsiooni analüüsi meetodit. Vastavalt sellele, millist biloogilist materjali kasutatakse mikrokiibile sidumiseks, nimetatakse neid spetsiifilisemalt näiteks DNA, oligonukleotiidide, raku, koe, valgu, antikeha, peptiidi jne. mikrokiipideks.

Mikrokiibi koostamise põhimõtte järgi on tagatud väga lihtne võimalus muuta kandja pinnale seotud oligonukleotiidide maatriksi (peptiidide vms.) koosseisu: Sellisel tagatakse suur kõnelause analüüsi platvormi paindlikkus väga erinevatele eesmärkidele vastavaks disainimisel ja seega on kõnealune tehnoloogia hõlpsalt rakendatav molekulaardiagnostika meetodina, eriti silmas pidades selle tehnoloogia maksumust võrrelduna mitmete teiste mikrokiibil põhinevate testidega. Kuigi kommertsiaalselt kättesaadavatel tehnoloogiatel on ka rida eeliseid (ostad ja kasutad etteantud piirides), säilib endiselt vajadus kiirelt ja spetsiifiliselt koostada erinevaid uusi DNA analüüsi- ja diagnoosikiipe vastavalt kiirelt muutuvale informatsioonile mille järgi igal nädalal avastatakse genoomis uusi riskialleele ja haiguste tekkes osalevaid mutatsioone.

Tänaseks on rutiinselt kasutusel juba mitmed erinevad mikrokiibi valmistamise meetodeid ning valik nende vahel sõltub lisaks rakenduse spetsiifikale peamiselt olemasolevatest vahenditest ja eelistustest. Kõige enamlevinum ja odavam on nii DNA oligonukleotiide, kui ka DNA sünteesi alustamiseks vajalike praimerite pipeteerimine vastava roboti abil kahesuunalise maatriksina kandjale, milleks on tavaliselt valgusmikroskoopias kasutuselolev alus- või katteklase. Selle meetodi puuduseks on ajakulu, mida on vaja olukorras kus on vaja asendada mõni oligonukleotiide primer teise, paremini sobivamaga. Uue primeri tellimiseks ja sünteesimiseks kulub päevi või isegi nädalaid. Primerite ümberdisainimise on lisafinantse nõudev ning lisaks juba olemasolev ning mitesobivaks osutunud primer jääb sisuliselt 99% ulatuses kasutamata.

Käesolevas doktoritöös on kasutatud mikrokiipide valmistamiseks teist, täiesti uut põhimõtet. Kõik vajalikud oligonukleotiidid sünteesitakse paralleelselt otse kiibile, milleks kasutatakse firmas Febit biomed GmbH poolt ehitatud integreeritud aparati "Geniom One" (Febit biomed GmbH, Heidelberg, Saksamaa). Siin kasutatakse oligonukleotiidide sünteesiks valgustundlike rühmadega protekteeritud fofforamidiide, mille seondumissaidid aktiveeritakse vajalikul ajal ja väga täpselt maatriksis mikropeeglite abil (sarnaselt slaidiprojektori tööpõhimõttele). Nii on võimalik sünteesida oligopraimereid mõlemas, nii 5'-3' kui ka 3'-5' suunas. Esimene sünteesi suund on vajalik Arrayed Primer Extension ehk APEX reaktsiooni läbiviimiseks, sest siin on vajalik vaba 3'-OH rühm DNA polümeraasi jaoks) ja teine esitatud sünteesi

suund on leidnud enam kasutamist nukleinhapete hübridisatsiooni puhul kasutatavates meetodites, milles ensümaatilist reaktsiooni ei toimu.

”Geniom One” aparati, milles on tegelikult terve praimerite sünteesi, reaktsiooni läbiviimise ja signaali analüüsi süsteem, kasutati kolme erineva funktsiooniga mikrokiibi disainimiseks. Esimesed kaks olid 3’–5’ suunas *in situ* sünteesitud mikrokiibid:

a) genotüpiseerida heinapalavikuga assotsieeruvaid geene hübridisatsiooni-meetodiga

b) detekteerida samaaegselt 81 erinevat Inimese Papillom viiruse alatüüpi.

Kolmanda mikrokiibi sünteesiks kasutati vastupidises ehk 5’- 3’ suunas *in situ* sünteesitud oligonukleotiidide, et APEXi reaktsiooni käigus analüüsida mutatsioone ja ühenukleotiidseid polümorfisme (SNP) ABCR geenis. Olles kord teinud kindlaks parimad oligonukleotiidide praimerite järjestused mikrokiibi analüüsi jaoks on võimalik sama maatriks üle kanda robotiga pipeteerimistehnoloogia abil valmistatavatele mikrokiipidele, mida saab toota massiliselt. *In situ* mikrokiibi sünteesitehnoloogia eeliseks pipeteerimistehnika ees on oligopraimerite maatriksi muutmiseks (ka oligopraimerite pikkus ja nukleotiidne koostis jt.) vajalik aeg (8–10 tundi) ehk see aeg, mille jooksul sünteesitakse ”Geniom One” abil uus mikrokiip. Puuduseks aga see ainult 1–8 mikrokiibi üheaegse sünteesi võimalus, mis on ideaalne uue kiibi väljatöötamisel, kuid ei sobi massanalüüsil haigla molekulaardiagnostika laboris.

Kuna mikrokiibi analüüsi abil soovitakse üheaegselt detekteerida aina suuremat arvu variatsioone, siis suureneb ka sellel toimuva analüüsireaktsiooni kompleksus. Seega kujuneb ühe mikrokiibi praimeritemaatriksi kavandamine multiparameetriliseks ülesandeks. Erinevate oligonukleotiidide tiheduse ühes spotis, praimeri seostumisalade asetuse tihedus kiibi maatriksis ja nende nukleotiidse järjestuse tõttu on vaja leida võimalikult parimad reaktsioonitingimused.

Käesolevas doktoritöös on esitatud ka reaktsionisüsteemi enda parameetrite nagu reaktsiooniruumi geomeetria ja analüüdi molekulide massiülekanne efekti mõju analüüsireaktsiooni (hübridisatsiooni) toimumise efektiivsusele. Kaheosalise hübridisatsiooni teooria mudeli (TCM) alusel on võimalik demonstree-rida, et tugevalt difusioonist mõjutatud analüüsitavate molekulide transport oligonukleotiidide praimeritele on sõltuv reaktsiooni lahuse kontsentratsioonist ja sellest johtuvalt lahuse viskoossusest ning lisaks mängib olulist rolli mikrokiibi pind, millele on immobiliseeritud oligonukleotiidide maatriks.

Seega tuleb mikrokiibi kavandamisel teha kindlaks kasutatavale süsteemile iseloomulikud analüüsi mõjutavad parameetrid, mis võimaldavad hõlpsamat optimaalsete reaktsioonitingimuste leidmist ja optimeerimist. See kõik on väga oluline aspekt mikrokiibi meetodi kasutamisel ja võimaldab standardiseerida geenianalüüsi protokollid reprodutseeruvate tulemuste saamiseks.

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## **PUBLICATIONS**

# CURRICULUM VITAE

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**1998–2000** *M.Sc* in Physical and Electrochemistry, Faculty of Chemistry, University of Tartu, Estonia  
**2002–2008** *Ph.D* student in the Institute of Molecular and Cell Biology, University of Tartu, Estonia

### Professional employment:

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**2001–2007** Institute of Molecular and Cell Biology, Department of Biotechnology, Tartu University, Estonian Biocentre, Scientist.  
**2002–2003** Division of Functional Genome Analysis, German Cancer Research Centre, Heidelberg, Germany, Visiting Scientist.  
**2004–2006** Division of Functional Genome Analysis, German Cancer Research Centre, Heidelberg, Germany, Visiting Scientist.  
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### Scientific Work:

I have been studying different aspects of the complexation reaction kinetics between trace metal ions and the polymeric acid molecules. Currently, my research projects have been associated with development of oligonucleotide

based microarrays for the DNA resequencing and genotyping methods using mainly the *in situ* synthesis platform for the production of oligonucleotide microarrays.

### List of publications

1. **Pullat, J.**, Kusnezow, W., Jaakson, K., Beier, M., Hoheisel, J.D., Metspalu, A. (2008) Mutational Analysis and SNP Typing by an Arrayed Primer Extension (APEX) Reaction on an *in situ* synthesized 5' - 3' Oligonucleotide Microarray. (accepted)
2. **Pullat, J.** and Metspalu, A. (2008) Arrayed primer extension reaction for genotyping on oligonucleotide microarray. In Methods in Molecular Biology: Prenatal Diagnosis: Preface. Ed by S. Hahn, L.G. Jackson. Prenatal Diagnosis (435), Series: Methods in Molecular Biology, 2008, Vol. 444 ISBN: 978-1-58829-803-4. Humana Press
3. **Pullat, J.**, Fleischer, R., Becker, N., Beier, M., Metspalu, A. and Hoheisel, J.D. (2003) Optimization of candidate-gene SNP-genotyping by flexible oligonucleotide microarrays; analyzing variations in immune regulator genes of hay-fever samples. BMC Genomics, 8, 282–.
4. Schliwka, J., **Pullat, J.**, Devilliers, E.-M., Hoheisel, J.D., Beier, M. (2007) Mit der Geniom-Microarray-Technologie HPV-Genotypen auf der Spur Biospectrum, 1, 52-53.
5. Bauer, A., Beckmann, B., Busold, C., Brandt, O., Kusnezow, W., **Pullat, J.**, Aign, V., Fellenberg, K., Fleischer, R., Jacob, A., Frohme, M. and Hoheisel, J.D. (2003) Use of complex DNA- and antibody-microarrays as tools in functional analyses. Comp. Funct. Genom. 4, 520–524.
6. Keis, H., Kruusmaa, J., and **Pullat, J.** (2000). Polarography and stripping voltammetry of lead-polycarboxylate complexes oligonucleotide dropping mercury and rotating disc electrodes; Proceedings of the Estonian Academy of Sciences. Chemistry. 49, No. 3, 156–167.

# ELULOOKIRJELDUS

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### Teadustegevus:

Eelnevalt olen uurinud raskete metalli-ioonide ja polümeersete hapete vahelise kompleksimoodustumise reaktsiooni kineetikat polarograafiliste meetodie abil.

Edaspidi olen seotud olnud projektidega, mille eesmärgiks on erinevate funktsioonidega *in situ* sünteesitud oligonukleotiidide mikrokiipide disain ja arendus.

## Publikatsioonid

1. **Pullat, J.**, Kusnezow, W., Jaakson, K., Beier, M., Hoheisel, J.D., Metspalu, A. (2008) Mutational Analysis and SNP Typing by an Arrayed Primer Extension (APEX) Reaction on an *in situ* synthesized 5' - 3' Oligonucleotide Microarray. (accepted)
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