

Minisequencing on oligonucleotide microarrays: comparison of immobilisation chemistries

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ABSTRACT

In the microarray format of the minisequencing method multiple oligonucleotide primers immobilised on a glass surface are extended with fluorescent ddNTPs using a DNA polymerase. The method is a promising tool for large-scale single nucleotide polymorphism (SNP) detection. We have compared eight chemical methods for covalent immobilisation of the oligonucleotide primers on glass surfaces. We included both commercially available, activated slides and slides that were modified by ourselves. In the comparison the differently derivatised glass slides were evaluated with respect to background fluorescence, efficiency of attaching oligonucleotides and performance of the primer arrays in minisequencing reactions. We found that there are significant differences in background fluorescence levels among the different coatings, and that the attachment efficiency, which was measured indirectly using extension by terminal transferase, varied largely depending on which immobilisation strategy was used. We also found that the attachment chemistry affects the genotyping accuracy, when minisequencing on microarrays is used as the genotyping method. The best genotyping results were observed using mercaptosilane-coated slides attaching disulfide-modified oligonucleotides.

INTRODUCTION

Single nucleotide polymorphisms (SNPs), which represent the most abundant form of genomic sequence variation, occur at about one out of a thousand nucleotides in the human genome (1,2). Methods with high throughput are required to analyse the genomic sequence variation between individuals in more detail and on a large scale. Miniaturised assays in a microarray (DNA chip) format offer the potential for genotyping of large numbers of SNPs at low cost (3). For DNA polymerase-assisted 'minisequencing' in the microarray format, detection primers annealing immediately 5' of each variable SNP in the sample DNA are immobilised through their 5'-end, followed by primer extension with single labelled nucleotide analogues that are complementary to the variable nucleotides using a DNA polymerase (4,5). Primer extension is currently gaining

acceptance as the reaction principle of choice for genotyping SNPs in microarray assay (6–12).

Microarrays of oligonucleotides can be produced at very high density by combinatorial light-directed *in situ* synthesis on solid surfaces (13; Affymetrix GeneChips®). These arrays, originally intended for comparative DNA sequencing using hybridisation to allele-specific oligonucleotide probes, cannot be directly applied in DNA polymerase-assisted reactions, since the oligonucleotide synthesis proceeds in the 3'–5' direction, and the 3'-ends of the oligonucleotides are not free to be extended. A recent microarray-based method utilises 3'–5' *in situ* synthesised complementary 'tag' oligonucleotides as capturing probes for minisequencing primer extension products formed in solution with primers carrying 5'-'tag' sequences (6). Oligonucleotides can also be synthesised *in situ* in the 5'–3' direction, but this approach seems to give a lower yield than the 3'–5' synthesis (14). Using a refined chemical strategy, *in situ* synthesised 3'–5' oligonucleotides can be inverted on a solid support to give the opposite polarity with a free 3'-end for DNA polymerase-mediated primer extension (15).

The most generally accessible approach for producing oligonucleotide microarrays is to synthesise the individual oligonucleotides separately prior to immobilisation on the solid surface. In this case the oligonucleotide is modified with a functional group that allows covalent attachment to a reactive group on the surface. Oligonucleotides modified with an NH₂-group can be immobilised onto epoxy silane-derivatised (16) or isothiocyanate-coated glass slides (17). Succinylated oligonucleotides can be coupled to aminophenyl- or aminopropyl-derivatised glass slides by peptide bonds (18), and disulfide-modified oligonucleotides can be immobilised onto a mercaptosilanised glass support by a thiol/disulfide exchange reaction (19). Alternatively, unactivated microscope slides may be used with activated, silanised oligonucleotides (20). Many of the attachment strategies are based on heterobifunctional crosslinking molecules, giving many alternatives to the linking molecule and to the modification of the oligonucleotide (17,21,22). Recently, chemically pre-activated microscope slides have become commercially available, increasing the number of options for attaching oligonucleotides in practice. With a few exceptions (21,23), the available attachment strategies have not been systematically compared for their performance in assays on microarrays.

To improve the fluorescent signal intensities, and hence the genotyping power and throughput of our minisequencing

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Table 1. Efficiencies of the reaction between the functional 5' groups on the oligonucleotide primers and the reactive groups on the microscope slides

Slide type	5'-modification on primer	Functional group on slide	Relative signal intensity ^a
Isothiocyanate	NH ₂ -(CH ₂) ₆ -O-(PO) ₂ -oligonucleotide	-N=C=S	1.0
'SuperAldehyde'	NH ₂ -(CH ₂) ₆ -O-(PO) ₂ -oligonucleotide	-CHO	0.090 ^b , 0.20 ^c
3D-Link™	NH ₂ -(CH ₂) ₆ -O-(PO) ₂ -oligonucleotide	long-chain hydrophilic polymer containing amine-reactive groups	7.7
Mercaptosilane	HO-(CH ₂) ₆ -S-S-(CH ₂) ₆ -O-(PO) ₂ -oligonucleotide H ₂ C=CH-CO-NH-R-oligonucleotide ^d	-SH	1.9
EZ-RAYS™	HO-(CH ₂) ₆ -S-S-(CH ₂) ₆ -O-(PO) ₂ -oligonucleotide H ₂ C=CH-CO-NH-R-oligonucleotide ^d	three-dimensional with -SH groups	0.19
Unmodified	(OCH ₃) ₃ -Si-R-oligonucleotide ^e	none	0.80

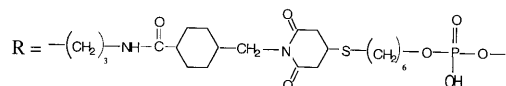
^aRelative signal intensities after terminal deoxynucleotidyl transferase-mediated extension of immobilised oligonucleotides were calculated from the average value for the 16 or 24 oligonucleotide spots on each slide. The signal intensities on each slide were compared pairwise to the signal intensities on the isothiocyanate-coated slides, which were given the value 1 in each comparison reaction, and the signal intensities on the other slides are normalised with respect to this value.

^bSignal intensity for 'SuperAldehyde 1', see Materials and Methods.

^cSignal intensity for 'SuperAldehyde 2', see Materials and Methods.

^dThe data were not available for the linker molecule.

^e



assays, we tested different immobilisation procedures for the oligonucleotide primers. We compared six types of differently activated glass slides (Table 1) with respect to the background fluorescence, the efficiency of the attachment reaction measured indirectly using terminal transferase-mediated extension reaction, and most importantly, the signal to noise ratios after performing minisequencing reactions with fluorescence labelling on the microarrays. We found that the efficiency of attaching the oligonucleotides to the glass slides depends largely on which chemistry is used.

MATERIALS AND METHODS

Samples and SNPs

DNA was extracted from blood samples of two volunteers by standard procedures. Six oligonucleotide primers defining the coding (co) and the non-coding (nc) DNA strands of three SNPs were used to analyse the DNA samples. Two of the SNPs were from the human angiotensin II type 1 receptor (AGTR1) gene (DDBJ/EMBL/GenBank accession no. AF245699; 24) and they are denoted AGTR1 1166co, AGTR1 1166nc, AGTR1 1062co and AGTR1 1062nc. The third SNP was from the human endothelial nitric oxide synthase (eNOS) gene (DDBJ/EMBL/GenBank accession no. X76307; 25) and is denoted eNOSco and eNOSnc. The samples were genotyped by solid-phase minisequencing in a microtitre plate format (26). The genotype of sample 1 was AGTR1 1166 CC, AGTR1 1062 AA, eNOS GG and that of sample 2 was AGTR1 1166 AA, AGTR1 1062 AG, eNOS GT. The alleles are denoted according to the variable nucleotide on the coding strand of the DNA.

Preparation of microarrays

The isothiocyanate surface activation was performed on standard microscope glass slides (Menzel-Gläser, Braunschweig,

Germany). NH₂-modified oligonucleotides (Tables 1 and 2) (Interactiva Biotechnologie GmbH, Ulm, Germany) were attached as described by Guo (17), except that 3-aminopropyltriethoxysilane (Sigma) was used for silanisation instead of the methoxy-derivative. The oligonucleotides were dissolved in 400 mM sodium carbonate buffer (pH 9.0) to a final concentration of 25 μM prior to spotting. Immediately after spotting, the slides were exposed to vaporised ammonia for 1 h, followed by three washes in distilled water. The arrays were stored at 10°C until use.

NH₂-modified oligonucleotides were attached to the aldehyde-modified 'SuperAldehyde'-slides (Tables 1 and 2) (TeleChem International Inc., Sunnyvale, CA; lot numbers 000529 and 000831) according to the protocol given by the manufacturer. The oligonucleotide concentration was 25 μM in 0.5× ArrayIt Micro spotting solution (TeleChem International Inc., Sunnyvale, CA). To remove the unbound oligonucleotides after spotting and deactivate the excessive aldehyde groups, the slides were processed as described in the protocol: washed twice in 0.2% sodium dodecyl sulfate (SDS) for 5 min each, twice in dH₂O for 5 min each and once in a solution containing 0.25 g Na₂BH₄ dissolved in 75 ml phosphate-buffered saline (PBS) and 25 ml EtOH for 5 min. Additionally the slides were washed three times in 0.2% SDS for 1 min each and twice in dH₂O for 1 min each. These slides were denoted 'SuperAldehyde 1'. We devised a modification of this protocol in which the slides were incubated in 0.1 M Tris-HCl pH 8.0 with 0.05 M NaCNBH₃ and 0.01 M NaOH for 15 min. The slides were then washed three times in 0.2% SDS for 1 min each and once with dH₂O. These slides were denoted 'SuperAldehyde 2'. Both 'SuperAldehyde' slides were dried and stored at 20°C.

NH₂-modified oligonucleotides were attached to the 3D-Link™-slides (Tables 1 and 2) (SurModics, Inc., Eden Prairie, MN) according to the protocol given by the manufacturer. The oligonucleotides were dissolved in 150 mM sodium phosphate buffer pH 8.5 at a 25 μM concentration. After printing the

Table 2. PCR and minisequencing primers

Locus	Primer ^a	Sequence, 5' to 3'	Nucleotide variation
AGTR 1 1166	5' PCR primer	GCG GTC CCA AAA GGG TCA GTC CCC AAA AGC CAA ATC CCA C	
	3' PCR primer	TTC TAA TAC GAC TCA CTA TAG AGA AAA GTC GGT TCA GTC CAC	
	ms primer co ^b	CAC TTC ACT ACC AAA TGA GC	A/C
	ms primer nc ^c	CAA TTC TGA AAA GTA GCT AA	T/G
	ms primer nc*	CCTT CAA TTC TGA AAA GTA GCT AA	
AGTR 1 1062	5' PCR primer	GCG GTC CCA AAA GGG TCA GTC CCC AAA AGC CAA ATC CCA C	
	3' PCR primer	TTC TAA TAC GAC TCA CTA TAG AGA AAA GTC GGT TCA GTC CAC	
	ms primer co	TCC ACC AAG AAG CCT GCA CC	A/G
	ms primer nc	TGT CAC TCA ACC TCA AAA CA	T/C
eNOS	5' PCR primer	GCG GTC CCA AAA GGG TCA GTC ATT CAG CAC GGC TGG ACC	
	3' PCR primer	TTC TAA TAC GAC TCA CTA TAG TGC TCC AGG GGC ACC TCA A	T/G
	ms primer co	CTG CTG CAG GCC CCA GAT GA	A/C
	ms primer nc	AGA AGG AAG AGT TCT GGG GG	

^aThe minisequencing primers contained a spacer-sequence of 15 T-residues in their 5'-ends.

^bco = detects coding strand of the DNA.

^cnc = detects non-coding strand of the DNA.

arrays were stored for 4–72 h in a chamber with 75% relative humidity (SurModics, Inc., Eden Prairie, MN). The excess amine-reactive groups were deactivated for 15 min at 50°C in a solution containing 50 mM ethanolamine, 0.1 M Tris-HCl pH 9.0 and 0.1% SDS. The slides were washed with dH₂O, in a solution containing 4× SSC and 0.1% SDS at 50°C for 15–60 min and finally with dH₂O. The slides were stored desiccated at 20°C until use.

The oligonucleotides attached to the mercaptosilane-derivatised slides (Orchid Bioscience, Inc., Princeton, NJ) were modified with 5' disulfide groups (see Tables 1 and 2) (Operon Technologies, Inc., Alameda, CA) and diluted to a concentration of 25 μM in 0.5 M sodium carbonate buffer (pH 9.0) and 0.02% SDS. After spotting, the slides were kept in a chamber with 75% relative humidity for 5 min to overnight. The slides were then washed three times with a solution containing 10 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween-20. After washing with dH₂O the slides were left to dry and stored at 20°C until use. For attaching acrylamide-modified oligonucleotides (Tables 1 and 2) (Sigma-Genosys Ltd, Cambridge, UK) to the mercaptosilane-surfaced slides the oligonucleotides were dissolved as for attachment to the EZ-RAYSTM-slides (Mosaic Technologies, Waltham, MA), see below. The post-spotting procedures were the same as those described for the disulfide-modified oligonucleotides.

Acrylamide-modified oligonucleotides were attached to the EZ-RAYSTM-slides (Tables 1 and 2) according to the December 01, 2000 version of the protocol given by the manufacturer. The oligonucleotides were dissolved in 100 mM sodium carbonate buffer (pH 10.0) containing 0.0008% *N*-lauroylsarcosine to a final concentration of 25 μM. The latent thiol groups on the slides were activated before spotting the oligonucleotides for 15–30 min in a solution containing 0.64 g of tris(carboxyethyl)phosphine hydrochloride in 45 ml of dH₂O.

The slides were briefly washed with dH₂O and dried at 20°C, the oligonucleotides were spotted and the arrays were left at 20°C for at least 60 min after arraying. The post-array processing included soaking the arrays for 30 min in 40 ml quench buffer containing sodium acrylate (Mosaic Technologies, Waltham, MA) and washing the arrays twice for 5 min each in 10 mM Tris-HCl pH 8.0 with 1 mM Na₂EDTA. Finally the arrays were rinsed briefly with dH₂O. The slides were stored at 20°C until use. Disulfide-modified oligonucleotides were also attached to the EZ-RAYSTM-slides. These oligonucleotides were diluted to a concentration of 25 μM, in a 0.5 M sodium carbonate buffer (pH 9.0) and 0.02% SDS. The post-spotting procedures were the same as those described for attaching the acrylamide-modified oligonucleotides to the EZ-RAYSTM-slides.

Silanised oligonucleotides (Interactiva Biotechnologie GmbH, Ulm, Germany) were immobilised to the unmodified glass surface (Menzel-Gläser, Braunschweig, Germany) (Tables 1 and 2) as described by Kumar *et al.* (20) with a few modifications. The slides were not washed before spotting since that caused the spots to spread and mix with each other. Finally the slides were air-dried and stored at 20°C until use. The concentration of the oligonucleotides was 15 μM and they were dissolved in DMSO/H₂O 2:1 (eNOS co) and 1:1 (eNOS nc).

Printing the oligonucleotides

A custom-built, modified industrial robot (Isel, Eiterfeld, Germany) with two TeleChem CMP2 (Sunnyvale, CA) printing pins controlled by an MCM-310 operating system and NUMO-6.0 software (Merval, Pietarsaari, Finland) was used to print the oligonucleotide detection primers onto the coated slides. The oligonucleotide spots were 125–150 μm in diameter and the centre to centre distance between two adjacent spots

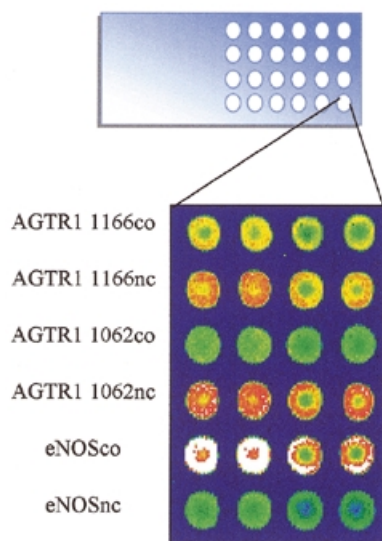


Figure 1. The microarray format with 24 subarrays of 4 mm in diameter on a microscope slide. The subarray in the figure with each oligonucleotide primer as quadruplicate was printed on a 3D-Link™-slide and scanned after terminal deoxynucleotidyl transferase extension of the primers using tetramethylrhodamine-labelled dATP.

was 250 μm . The isothiocyanate-, aldehyde-, 3D-Link™- and mercaptosilane-arrays consisted of six oligonucleotide primers (AGTR1 1166co, nc, AGTR1 1062co, nc, eNOSco and nc), each of which was spotted as quadruplicates per well (Fig. 1). The EZ-RAYS™- and unmodified slides were prepared by spotting two oligonucleotides (eNOS co, eNOS nc) at eight positions per well, as were the acrylamide-modified oligonucleotides onto the mercaptosilane-surfaced slides.

Terminal deoxynucleotidyl transferase reactions

The arrays were pre-heated to 37°C in a custom-made aluminium reaction rack. A re-usable silicon rubber grid was applied on the arrays to form 80 separate reaction wells on each slide (10). Ten microlitres of a reaction mixture containing 0.5 μM tetramethylrhodamine (TAMRA)-labelled dATP (NEN™ Life Science Products, Brussels, Belgium), 0.3 μM unlabelled dATP, 0.5 U/ μl terminal deoxynucleotidyl transferase (MBI Fermentas GMBH, St Leon-Rot, Germany) and 1 mM CoCl_2 in 0.2 M potassium cacodylate pH 7.2, 0.1 mM DTT and 0.01% Triton X-100 was added to each reaction well. The extension reaction was allowed to proceed for 30 min at 37°C after which the slides were washed with dH_2O , in a solution of 90 mM sodium citrate, 900 mM NaCl, 0.05% *N*-lauroyl-sarcosine and finally with dH_2O .

PCR amplification of SNPs

The PCR primers were designed based on the sequences of the AGTR1 and eNOS genes and synthesised by Interactiva Biotechnologie GmbH (Ulm, Germany). Their sequences are listed in Table 2. DNA fragments spanning the SNP sites were amplified in a Programmable Thermal Controller (MJ Tetrad Research, Watertown, MA) using 20 ng of genomic DNA with 3.5 U AmpliTaq Gold DNA polymerase (Perkin-Elmer,

Branchburg, NJ) and 200 μM dNTPs in 100 μl of DNA polymerase buffer supplied with the enzyme (N808-0244). The SNP markers AGTR1 1062 and 1166 were amplified within one PCR fragment with the PCR primers at 0.2 μM concentration and the SNP marker eNOS was amplified with primers at 0.5 μM concentration. The PCR parameters were: initial activation of the polymerase at 95°C for 10 min, then 35 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 7 min.

Minisequencing on oligonucleotide arrays

The PCR products from each sample were combined and precipitated by ethanol, followed by suspension into 40 μl of dH_2O . The arrays were pre-heated to 37°C in the reaction rack and the PCR products were denatured for 1.5 min at 95°C and placed on ice. Ten microlitres of a buffer containing 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 1 M NaCl was added. Ten microlitres of this mixture was applied to four reaction wells of 3 mm in diameter on the pre-heated arrays. The annealing reaction was allowed to proceed in a humid chamber at 37°C for 15 min. The arrays were washed briefly with a solution of 5 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 100 mM NaCl, 0.1% Triton X-100.

Each minisequencing reaction mixture contained one of the four TAMRA-labelled ddATP, ddCTP, ddGTP, ddTTP analogues at a 0.75 μM concentration (NEN™ Life Science Products, Brussels, Belgium) together with the other three unlabelled ddNTPs at a concentration of 0.5 μM in 26 mM Tris-HCl pH 9.5, 6.5 mM MgCl_2 , 0.2% Triton X-100 buffer with 0.075 U/ μl DynaSeq DNA polymerase (gift from Finnzymes OY, Helsinki, Finland) or ThermoSequenase (AP Biotech, Uppsala, Sweden). The arrays carrying the annealed templates were pre-heated to 68°C, and 20 μl of reaction mixture was added to four separate reaction wells. The reaction was allowed to proceed at 68°C for 5 min in a humid chamber, after which the slides were washed with dH_2O , washed once with 50 mM NaOH for 2 min and washed with dH_2O . The slides were further washed twice for 5 min at 65°C in a solution containing 3 mM sodium citrate, 30 mM NaCl and 0.1% SDS (pH 7.0) and finally with dH_2O .

Signal detection and data analysis

Fluorescence signals were measured using an array scanner (ScanArray® 5000, GSI Lumonics, Watertown, MA) and the signal intensities were determined with the QuantArray® analysis software (GSI Lumonics). The excitation laser was Green HeNe, 543.5 nm. The laser power and photomultiplier tube (pmt) gain were adjusted according to the signal intensity. The isothiocyanate-coated slides served as reference slides to which the other slides were compared. The signal from each spot was corrected for the average background in each well, measured from six spots immediately below the array, by subtraction, giving the signal intensity value. The genotypes were determined by calculating the ratio between the signal intensities from the reaction for one of the alleles divided by the signal from the other allele.

RESULTS AND DISCUSSION

To improve the genotyping specificity of our microarray-based fluorescent minisequencing system, we evaluated six

chemistries for covalent coupling of the oligonucleotide primers to glass microscope slides. The evaluation included isothiocyanate-coated slides (17), aldehyde-coated slides and 3D-link™-slides with a three-dimensional hydrophilic surface, all attaching amino-modified oligonucleotides, mercaptosilane-slides attaching disulfide-modified oligonucleotides (19), EZ-RAYS™-slides with three-dimensional coating and latent thiol groups attaching acrylamide-modified oligonucleotides, and unactivated slides attaching sianised oligonucleotides (20) (Table 1). For the aldehyde-coated 'SuperAldehyde'-slides we tested two deactivation procedures, and to the EZ-RAYS™-slides and the mercaptosilane-slides we attached oligonucleotides modified both with acrylamide and disulfide residues.

Previously, at an early stage for microarray-based methods, when no commercially available slides or fluorescent scanners were available, we tested three possibilities for covalent attachment of oligonucleotides to microscope glass slides (16–18). These experiments were done using radioactive labelling in the minisequencing reaction, followed by phosphorimager scanning. We found that isothiocyanate coating for attaching NH₂-oligonucleotides (17) gave the best signal to noise ratios in the assay and was the most reproducible of the three methods (K.Lindroos, M.Raitio and A.-C.Syvänen, unpublished results). Currently this attachment chemistry is used in our routine microarray-based genotyping assays (10,12). This method also served as the reference procedure in the study presented here.

Based on our previous experiments using isothiocyanate-coated and unmodified slides, oligonucleotide concentrations of 25 µM yielded a maximum attachment efficiency and the highest signal intensities in the minisequencing reaction (K.Lindroos, M.Raitio and A.-C.Syvänen, unpublished results). As this concentration was also recommended by the manufacturers of the commercial 'SuperAldehyde'-, 3D-Link™- and EZ-RAYS™-slides, as well as for the mercaptosilane-coated slides (19) and the unmodified slides in the literature (20), 25 µM primers were used for spotting the primer arrays.

Background fluorescence

Initially, the background fluorescence of the six differently derivatised microscope slides was measured before attaching the oligonucleotides onto them. The unmodified slide and the mercaptosilane-coated slide gave the lowest background fluorescence. The background fluorescence of the slides after performing the minisequencing reaction was measured outside the oligonucleotide primer spots, to evaluate the unspecific binding of TAMRA-labelled ddNTPs to the surface. The unmodified slide showed lower background fluorescence than all the coated slides. The 3D-Link™- and EZ-RAYS™-slides gave significantly higher background than the other slides when the average signal from all four bases was calculated (Fig. 2).

Attachment efficiency

The efficiency of coupling oligonucleotide primers to the slides coated with different functional groups was determined by immobilising sets of oligonucleotide primers modified in their 5'-ends with the appropriate reactive group (Table 1). The ends of the immobilised oligonucleotides were extended with TAMRA-labelled dATP using terminal deoxynucleotidyl

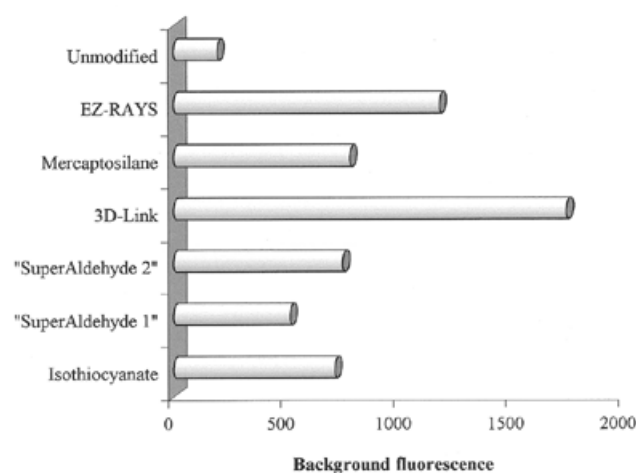


Figure 2. Comparison of background fluorescence for the different types of microscope glass slides after exposing the slides to minisequencing reaction mixtures containing TAMRA-labelled ddNTPs. The figure shows the average background from all four bases from two experiments after measurement under the Green HeNe laser. The laser power during the measurements was 95% and the pmt gain was 80%. The background fluorescence is given as arbitrary fluorescence units.

transferase, followed by measurement in the fluorescence array scanner. The fluorescence signals from the oligonucleotides on each slide type were compared with the corresponding signals on the isothiocyanate-modified slide. For all the oligonucleotides included in the experiment, the 3D-Link™-slides gave higher signal intensities than the isothiocyanate slides, and a similar result was observed with the mercaptosilane-coated slides. The other surfaces appeared to attach less oligonucleotides than the isothiocyanate-coated slides (Table 1).

Both the activated EZ-RAYS™- and mercaptosilane-slides are based on thiol groups mediating the binding of the oligonucleotides to the surface. Thus both acrylamide- and disulfide-modified oligonucleotides may be attached to these slide types. We tested coupling acrylamide-modified oligonucleotides onto the mercaptosilane-slides, and disulfide-modified oligonucleotides onto the EZ-RAYS™-slides, as can be seen in Table 1. The mercaptosilane-surfaced slides gave a mean signal intensity for the acrylamide-modified oligonucleotides that was only 30% of that of the disulfide-modified oligonucleotides and the mean signal intensity for the disulfide-modified oligonucleotides was 90% of that of the acrylamide-modified oligonucleotides.

Minisequencing reactions

As the most relevant evaluation, the different slide types were tested for their performance as supports for minisequencing reactions. The signal intensities and signal ratios observed in the minisequencing reactions on each slide were compared with those on the isothiocyanate-slide using a sample that is homozygous at all variable sites as template. In this experiment the aldehyde-coated slides repeatedly gave signal intensities that were too low for reliable genotyping (data not shown). The 3D-Link™-slides gave signal intensities that were 6–40 times higher than those on the isothiocyanate-modified slides, but

Table 3. Minisequencing results on the 3D-Link™-slides, the mercaptosilane-coated slides, the EZ-RAYS™-slides and the unmodified slides compared with those on the isothiocyanate-coated slides

SNP		Signal intensity ^a (arbitrary fluorescence units)				Signal/noise ^b			
		3D-Link™	Isothiocyanate	Mercaptosilane ^c	Isothiocyanate	3D-Link™	Isothiocyanate	Mercaptosilane ^c	Isothiocyanate
AGTR1 1166co	C-allele	31 000	2500	21 000	8300	100	50	47	208
	A-allele	310	50	450	40				
AGTR1 1166nc	G-allele	12 000	280	9300	10	24	7.0	47	0.013
	T-allele	500	40	200	780				
AGTR1 1062co	A-allele	19 000	1400	21 000	7000	5.1	11	12	∞
	G-allele	3700	130	1800	0				
AGTR1 1062nc	T-allele	27 000	3000	21 000	6900	250	300	46	53
	C-allele	110	10	460	130				
eNOSco	G-allele	62 000	3500	60 000	24 000	370	88	38	4.8
	T-allele	170	40	1600	5000				
eNOSnc	C-allele	7300	1200	17 000	3600	12	7.1	21	17
	A-allele	590	170	830	210				
		EZ-RAYS™	Isothiocyanate	Unmodified ^d	Isothiocyanate	EZ-RAYS™	Isothiocyanate	Unmodified ^d	Isothiocyanate
eNOSco	G-allele	50	8500	6600	16 000	0.069	3.0	11	36
	T-allele	720	2800	610	440				
eNOSnc	C-allele	4800	8400	16 000	4100	69	26	7.0	32
	A-allele	70	320	2300	130				

^aSignal intensity = signal intensity of oligonucleotide spot – average background fluorescence. The average values were calculated from two different reactions using sample 1 as DNA template, which was homozygous at all SNP sites. The laser power was 85% for the 3D-Link™/isothiocyanate comparison and for the mercaptosilane/isothiocyanate comparison. For the other two comparison reactions the laser power was 95%. The pmt gain was 75% for the 3D-Link™/isothiocyanate comparison and 85% for all the other comparison reactions.

^bThe signal intensity ratio between the expected and the other possible allele.

^cThe AGTR1 1166 nc* oligonucleotide was used for the mercaptosilane-array whereas the isothiocyanate-array had the AGTR1 1166 co oligonucleotide (see Table 2).

^dThe oligonucleotide concentration was 15 μM for the unmodified oligonucleotides.

the genotyping was hampered by a high background from unspecifically bound fluorescent ddNTPs (Table 3). The mercaptosilane-modified slides gave two to five times higher signal intensities than the isothiocyanate-slides, and since the level of background fluorescence was low for these slides, the genotyping results were unequivocal (Table 3). As expected from the terminal deoxynucleotidyl transferase-mediated extension reaction, the other thiol-surfaced slides, the EZ-RAYS™-slides, did not give as high signal intensities as the mercaptosilane-slides, despite their three-dimensional coating. The signal intensities for the EZ-RAYS™-slides were at their best 50% of that of the isothiocyanate-slides. The unmodified slides gave similar signal intensities to the isothiocyanate-slides (Table 3).

Finally, the 3D-Link™-slides and the mercaptosilane-surfaced slides, which gave higher signal intensities than the isothiocyanate-surface, were compared with each other in a minisequencing reaction using sample 2, which was heterozygous at four of the six variation sites, as template. The average signal intensities were approximately two times higher for the 3D-Link™-slides than for the mercaptosilane-slides, except for the oligonucleotides AGTR1 1166nc* and AGTR1 1062nc, where the situation was reversed (results not shown). This observation could be due to differences in the efficiency

of the 5'-modification between the primers. However, a slightly better discrimination between heterozygous genotypes was observed on the mercaptosilane-coated slides than on the 3D-Link™-slides (Fig. 3). Comparison of signal intensity ratios from samples 1 and 2 demonstrates the high power of discrimination between homozygous and heterozygous genotypes.

CONCLUSIONS

We found that the amount of immobilised oligonucleotide gives a good estimate about how specifically the minisequencing reactions will function. We were able to determine that, in the minisequencing reactions, the two-dimensional mercaptosilane-derivatised glass slides gave the best genotyping ratios, although the 3D-Link™-slides with a three-dimensional coating gave higher signal intensities. However, we believe that further optimisation of both the attaching procedure and the reaction conditions itself would improve the results with the 3D-Link™-slides. Also, the unmodified slides attaching silanised oligonucleotides seemed to be a promising new alternative for attaching the oligonucleotides, mostly because of their very low background fluorescence.

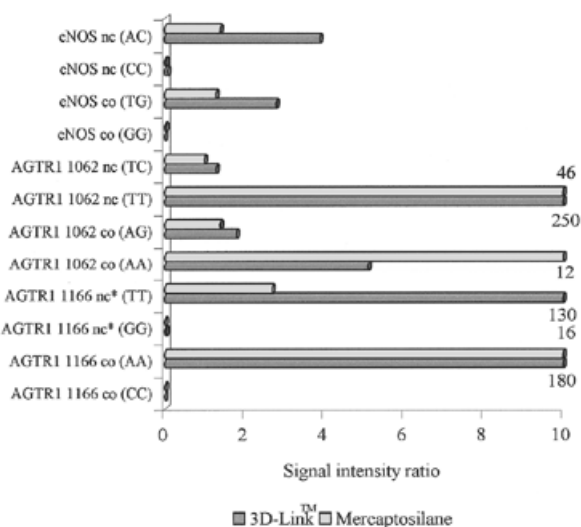


Figure 3. Signal intensity ratios from minisequencing reactions performed on the 3D-Link™-slides and mercaptosilane-coated slides from a sample that was homozygous at all sites and from a sample that was heterozygous at the AGTR1 1062 and eNOS SNPs and homozygous at the AGTR1 1166 SNP. The signal intensity ratios that were >10 are indicated with numbers.

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