Analysis of the factors affecting the accuracy of detection for single base alterations by oligonucleotide microarray

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Abstract

The oligonucleotide microarray, a high-throughput polymorphism detection technology, holds great promise for the characterization of complex genetic variance. To achieve greater sensitivity and specificity for it to be an effective platform technology we present results and discuss some of the factors influencing signal intensities and single-mismatch discrimination in array-based mutation/SNP detection. Probes with a series of concentrations were spotted onto the slide in order to find the optimal concentration with the identifiable satisfying signals and the stable ratios between matched and mismatched probes. It was found that under our experimental conditions, when the initial probe concentration is higher than the maximum immobilization capability of the slide (7.5 μM), the hybridization signal will be saturated and the ratio between matched and mismatched probes will be more stable than at a lower probe concentration. Considering the cost of probes and the systematic stability, a constant spotting concentration of 10 μM was selected. The stability of different types of mismatched oligo-DNA duplexes on the glass surface was also confirmed. The results show that the order of stability of mismatched oligo-DNA duplexes on a glass surface is in general agreement with previous reports conducted using liquid and polyacrylamide gel pads. This suggests that the influence of the mismatched base pair on the stability of the duplex in a solid hybridization system is similar to that in the solution hybridization environment.

Keywords: mutation; oligonucleotide array sequence analysis; polymorphism, single nucleotide

Introduction

In order to achieve rapid analysis of mega-quantities of samples with greater accuracy and sensitivity in mutation/SNP discovery and genotyping, there is a definite need for development of a sensitive oligonucleotide microarray platform technology to meet the challenge of advancements in biological sciences and medicine. Presently, a variety of techniques are available for new mutation/polymorphism identification, such as RFLP, direct sequencing, single-strand conformation polymorphism (SSCP) analysis, chemical or enzymatic mismatch cleavage detection, denaturing gradient gel electrophoresis, denaturing HPLC, TaqMan genotyping, and allele-specific ligation (Shi, 2001; Weiner and Hudson, 2002). Most techniques can be broadly classified as either hybridization-based or enzyme-based. Recent development, called the DNA microarray, is a hybridization-based genotyping technique that offers simultaneous analysis of many polymorphisms (Guo *et al.,* 1994; Lipshutz *et al.,* 1995; Hacia *et al.,* 1996; Drmanac *et al.,* 2000). Perfectly matched sequences hybridize more efficiently to their corresponding oligomers on the array, therefore giving stronger fluorescent signals over mismatched probe-target combinations. The advantage of this method is that a large collection of small PCR products can be scanned in one hybridization. Despite the theoretically high levels of scanning capacity, this application suffers some significant limitations in detecting polymorphisms. Recent experiments using microarrays demonstrated only a 97% accuracy (Patil *et al.,* 2001; Kirk *et al.,* 2002). Furthermore, high false positive rates of 11-21% have been observed with this technology, limiting its utility in both SNP and tumor analysis (Halushka *et al.,* 1999). In fact, the precision and the discrimination ability of this technology is dependent upon many factors including array fabrication, stringency of hybridization and strategies for designing probes and so on.

The rationale of oligo microarrays used to detect single nucleotide variance is that a single mismatched base pair can reduce the melting temperature (Tm)

of the hybrid by as much as $10^{o}C$ when the oligo-nucleotide is shorter than 20 nucleotides (Wallace *et al.,* 1979). However, thermodynamic studies have reported that not all mismatched base pairs have equal stability. G-T and G-A mismatches have been reported as the most stable duplexes. On the other hand, G to T mutation leading to C-T mismatch is one of the most unstable pairs (Werntges *et al.,* 1986; Ke and Wartell, 1993). Mismatches C-C due to G to C mutations have also been reported to significantly destabilize the duplexes (Peyret *et al.,* 1999). The order of stability of oligonucleotide-DNA duplexes with different mismatched bases at the same position as presented by Ikuta (Ikuta *et al.,* 1987) is (from most to least stable) T-A,A-T $>$ G-T,G-A $>$ A-A,T-T,C-T,C-A. Lee *et al.* (2004) presented a theoretical model, correlating well with previous experimental findings, which was considered as a guideline for single-point mutation probe design. However, his study was limit-ed to properties in solution. The use of a solid surface such as glass slides, however, would introduce sur-face effects. Even though independent experiments on a three dimensional surface, such as polyacryla-mide gel pads, have already proved the value of this model, other experiments especially conducted on the two dimensional surface are imperative for its vali-dation. Yet so far, whether similar nucleotide be-havior is performed on solid surfaces has not been reported. In this paper, we present results in detail on the influence of different types of mismatched base pairs on mutation/SNP discrimination and dis-cuss some other problems involved with the design of oligonucleotide microarrays.

Materials and Methods

Probe design

Probes were designed using the software, Primer Premier 5.0, and were then followed by manual checks. Melting temperature (*Tm*) of all probes cal-culated by the Equation 1 varied from $48^{o}C$ to $52^{o}C$. The allele-specific extension probes were synthesized by Takara Biotech with a 5' amino group that faci-litated their immobilization on the glass slide. A spacer sequence of 10 T residues was included at the 5' end of the gene-specific sequence. The poly-morphic sequence was situated near the center of each hybridization sequence.

$$Tm = 2(T+A)+4(G+C) \qquad \text{(Equation 1)}$$

G, C, A and T represent the numbers of guanine, cytosine, adenine and thymine bases respectively in the oligonucleotide (Anderson, 1999).

Oligo array fabrication

The amino modified oligonucleotides were spotted onto aldehyde slides (Shanghai Biostar Genechip, Inc.) using a Cartesian Prosys 5501A (Cartesian Te-

chnologies, Irvine, CA) fitted with ChipMaker Micro-Spotting Technology (TeleChem International, Sunny-vale, CA). Glass slides with immobilized probes were hydrated for 30 min in 70% humidity, and then dried for 30 min at room temperature. They were further soaked at room temperature in 0.2% SDS for 10 min and washed in distilled H_2O for 10 min twice. The slides were then dried again and ready for use.

The preparation of labeled PCR products

25 μl of PCR reaction system contained 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.2-0.5 ng/μl template, 0.4 μM Cy5-labeled primer at 5'-end (Synthesized by Takara Biotech) and 3 U rTaq DNA polymerase (Takara Biotech). The mixture was incubated for 2 min at $94^{o}C$ and 35 cycles of PCR were performed at $94^{o}C$ for 20 s, $60^{o}C$ for 20 s and $72^{o}C$ for 20 s, followed by a final extension at $72^{o}C$ for 5 min. And then 6 μl PCR Cy5-labeled products were used for hy-bridization.

Hybridization and washing

Fluorescent mixtures including PCR products were mixed with hybridization solution (5 × SSC, 0.2% SDS), denatured for 5 min at $95^{o}C$, and followed by incubation on ice immediately. Then denatured mix-tures were applied onto the microarray slide under a cover glass and placed in $42^{o}C$ chamber for 1 h. The temperature and the reaction time selected was found to be optimal for specificity and sensitivity of single-nucleotide variance analysis (data not shown). After hybridization, the microarray slide was washed at room temperature with washing solution (2 × SSC, 0.2% SDS solution) for 5 min and then dried at room temperature. Short hybridization time and low fluo-rescent signals, hybridization and washing conditions were optimized and simplified described above.

Signal detection and data analysis

The arrays were scanned with ScanArray 4000 (GSI Lumonics, Bellerica, MA). The acquired images were analyzed using GenePix 3.0.5 software (Axon Instru-ments, Inc). All of the spot signal intensities were recorded after subtracting the background intensity.

Results

Effect of oligonucleotide concentrations on signal intensity and signal ratios of matched/mismatched probes

To distinguish the relationship between the initial spotting concentration and the actual oligonucleotide density on the surface, different concentrations of oligonucleotide probes labeled with an amino group at the 5'-end and a Cy3 dye at the 3'-end, were printed on the glass surface for immobilization. The immobilization efficiency of oligonucleotide probes

was quantified by fluorescence scanning before and after washing. The results shown in Figure 1 indicate that the surface density increases with increased oligonucleotide concentration when the initial oligonucleotide concentration is less than 7.5 μM. The density of immobilized probes remains stable above the initial probe concentration of 7.5 μM. Several repeats of experiments with different probes yielded the similar results (data not shown). However, the analysis of the relationship between initial probe concentration and immobilized probe density is based on the assumption that fluorescent signals represent the density of immobilized probes on the slide. In order to substantiate this assumption and to determine whether the results of the hybridization experiments are in agreement with the above conclusion, we spotted two probes that had one single-base variance (probe C and probe T) with a series of concentrations from 0.08 μM to 30 μM on one slide. Each oligonucleotide probe was printed in quintuplicate and the intensities were averaged for each probes after hybridization with labeled PCR products of a heterozygote (TC), and homozygotes (TT) and (CC) respectively. The results shown in Figures 2A and 2B and
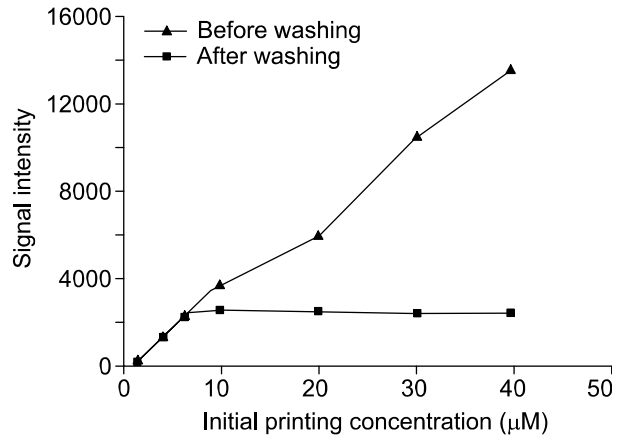

Figure 1. Effect of oligonucleotide probe concentrations on surface density of immobilized oligonuleotides. The spotted oligonucleotide probe was labeled with an amino group at its 5'-end and Cy3 dye at its 3'-end, and mixed with an identical sequence oligonucleotide which has an amino group labeled at its 5'-end (ratio of 1:10). Mixtures of oligonucelotide ranging from 2-40 μM were spotted on aldehyde glass slides, incubated at room temperature for 30 min and then washed with 0.2% SDS.

Table 1. The partial results of hybridization of different types of single base mismatch obtained from the p53 mutation detection.

Serials	Probes	Referential probes	Association type (liquid-solid)[a]	Match/mismatch ratio[b]
1	TACGCCAC*A*AGCTCCA		G-A	2.7 ± 1.4
2	TACGCCAC*T*AGCTCCA	TACGCCA*CC*AGCTCCA	G-T	2.9 ± 1.2
3	TACGCCA*A*CAGCTCCA		G-A	2.1 ± 1.4
4	TACGCCA*T*CAGCTCCA		G-T	4.3 ± 1.3
5	CTCCTCT*G*GACCTGC	CTCCTCT*T*GACCTGC	A-G	7.7 ± 2.4
6	CTCCTCT*C*GACCTGC		A-C	24.1 ± 4.9
7	ATGAACC*A*GAGGCCCA		C-A	8.6 ± 2.9
			C-T	9.5 ± 3.5
8	ATGAACC*T*GAGGCCCA	ATGAAC*CG*GAGGCCC	G-T	2.0 ± 1.3
9	ATGAAC*T*GGAGGCCCA		C-A	12.2 ± 3.6
10	TGAGG C*A*C TGCCCC		C-T	20.0 ± 4.4
11	TGAGG C*T*C TGCCCC	TGAGG *CG*C TGCCCC	G-T	1.9 ± 1.2
12	TGAGG *T*GC TGCCCC		G-G	1.8 ± 1.1
13	GCACAAACA*G*GCACCT		G-A	3.2 ± 1.2
14	GCACAAACA*A*GCACCT		G-T	2.1 ± 1.2
15	GCACAAACA*T*GCACCT	GCACAAACA*CG*CACCT		
16	GCACAAACAC*A*CACCT		C-A	11.3±2.6

[a]Means the associated order of nucleotides used in the table is solid-liquid, *e.g,* in C-A, C is the nucleotide at an immobilized probe, while A is the nucleotide at a liquid phase probe. [b]Means the ratio of the signal intensity of reference probes which was perfectly matched with the target to that of mismatched probes
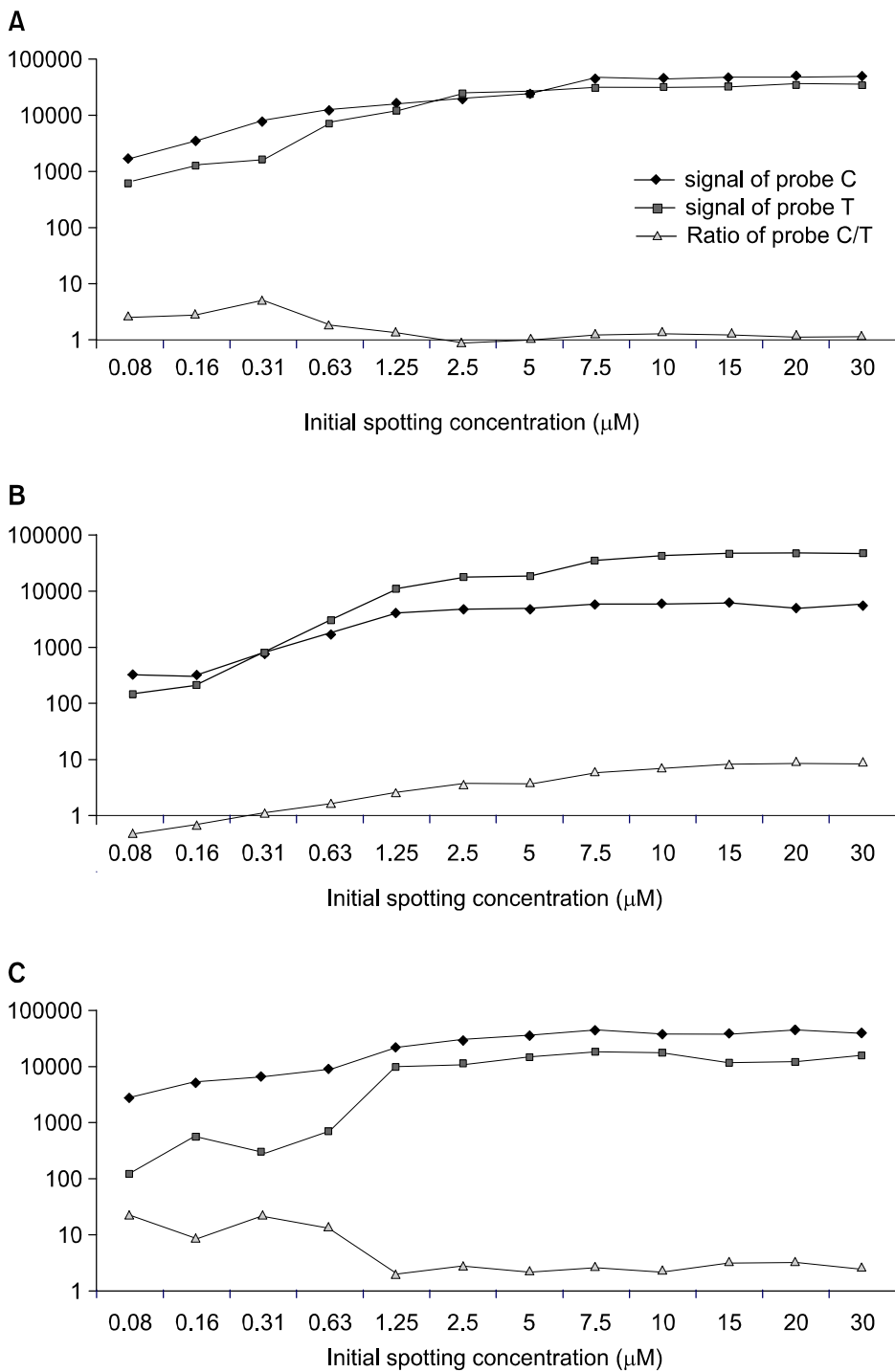
A

B

C

Figure 2. The signal intensities and signal ratios coming from the microarray hybridization. Two different single nucleotide probes (probe C: GTGAAGAACGACCTA AC; probe T: GTGAAGAA*T*GACC TAAC) in series of concentrations from 0.08 μM to 30 μM were spotted on the same array. Each oligonucleotide probe at a certain concentration was printed in quintuplicate and the intensities were averaged for each probe after hybridization to labeled PCR products with genotypes of CT (Figure 2A), TT (Figure 2B), and CC (Figure 2C) respectively.

2C suggest that the hybridization signal intensity is almost saturated and kept stable at concentrations above 5 μM, which leads to the ratio of two probes at concentrations between 7.5 μM and 30 μM being more stable than at lower concentrations. Therefore, the concentration itself was not the crucial factor for variance of signal intensities and signal ratios among the certain range of initial probe concentrations (7.5-30 μM). Under our experimental conditions, the initial concentration of 7.5 μM is close to the maximum immobilization capacity of the slide. Considering the cost of probes and the systematic stability, we selected a constant spotting concentration of 10 μM.

Effects of the base composition on distinguishability

Probes in Table 1 were designed to detect hotspot mutations of tumor suppression gene *p53* (NC_ 000017) in cancer cells. We designed a mismatched nucleotide close to the center of an oligonucleotide where it has been proven to have a strong dest- abilizing effect on the duplex. Each oligonucleotide probe was printed in quintuplicate and the intensities were averaged for each probe after hybridization with the labeled PCR product. Match/mismatch ratios in Table 1 are the average of ratios of triplicate experi- ments. Table 1 lists partial probes' data acquired through hybridization under our experimental con- ditions.

The kind of mismatched base pair of probe 1 and 3, resulting from mutation C to A, is a G-A mismatch, and that of 2 and 4, resulting from mutation C to T, is a G-T mismatch. The mismatched nucleotide sites on probes 1 and 3 are at the ninth and eighth base positions (from the 5'-end of the oligonucleotide) respectively. Likewise, there is analogous relationship between probes 2 and 4. The ratios of perfectly matched probe (Referential Probes) to probe 1, 2, 3, and 4 are 2.7 ± 1.4, 2.9 ± 1.2, 2.1 ± 1.4, 4.3 ± 1.3 respectively. Both the differences of ratios between probe 1 and 3 and that of ratios between probe 2 and 4 are not significant. Thus we could conclude that mismatched nucleotide position is a minor factor if located near the center of the oligonucleotide probe as the method we used to design probes.

From the data shown in Table 1, all match/mis- match ratios of G-T and G-A mismatches are ob- viously lower than those of C-T and C-A, which means that G-T and G-A mismatches slightly dest- abilized a duplex, while C-T and C-A mismatches significantly destabilize it. Our finding resulting from the experiments conducted on glass surface is in general agreement with the Ikuta's reports about the order of stability of oligo-DNA duplex (Ikuta *et al.,* 1987). Accordingly, the influence of the mismatched base pair on the stability of the duplex in the solid hybridization system is similar to that in the solution hybridization environment. In addition, we found that a G-G mismatch, like G-T and G-A, is also slightly destabilizing. The results of various kinds of single- base mutation probes hybridized to the same fluore- scently labeled target DNA can be seen in Figure 3. Though different kinds of mismatches give different stabilities of the oligo-DNA duplex, the overall ratio of match/mismatch probe is greater than 1.8 in general (data not shown).

Adding control probe for improving reliability

In Table 2, primers 1 and 2 are the PCR primers for amplifying the partial fragment of the CYP1A1 gene, Seq T and Seq C are the probes designed to detect its T and C alleles. SeqA is used as a negative control, it has an adenine residue at the mutation site. The results from Figure 4(A), 4(B) and 4(C), detecting genotypes of TT, TC and CC respectively, whose signal ratios of SeqT/SeqC are 13.5, 1.29 and 0.19 respectively (Table in Figure 4), show that two probes are enough to distinguish single nucleotide poly- morphisms. However, under abnormal hybridization conditions such as below the optimal temperature, the ratio of signal intensity of two probes cannot correctly reflect the actual gene polymorphism. From the result shown in Figure 4(D) which is obtained from the hybridization with the PCR of TT genotype, it can be
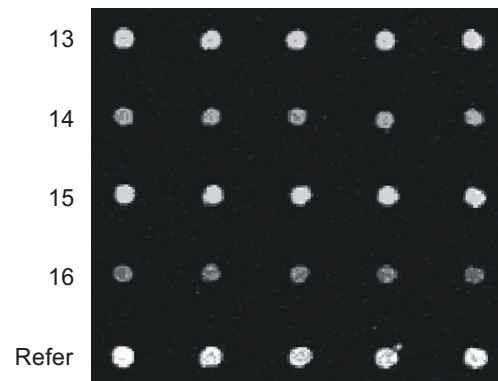

Figure 3. The results of hybridization of various kinds of single-base mutation probes. Probes 13, 14, 15, 16 and referential probe (Refer) hybridized to the same fluorescently labeled target DNA that is perfectly matched with the referential probe. The sequence of probes is listed correspondingly in Table 1. The order of signal intensity from high to low represented by pseudo-color is: white > green > blue.

Table 2. The primers and probes of *CYP1A1* (NT_024654) for SNP detection.

Alias	Sequence 5' to 3'	Length	*Tm*	Type	Nucleotide variation
Primer 1	GAGGTAGCAGTGAAGAGGTGTAGCCG	26	-	Forward primer	-
Primer 2	CTGAGGTGGGAGAATCGTGTGAGC	24	-	Reverse primer	-
Seq T	CACCTCC*T*GGGCTCA	15	50.0	Probe for T allele	T
Seq C	CACCTCC*C*GGGCTC	14	50.0	Probe for C allele	C
Seq A	CACCTCC*A*GGGCTCA	15	50.0	Probe as control	A
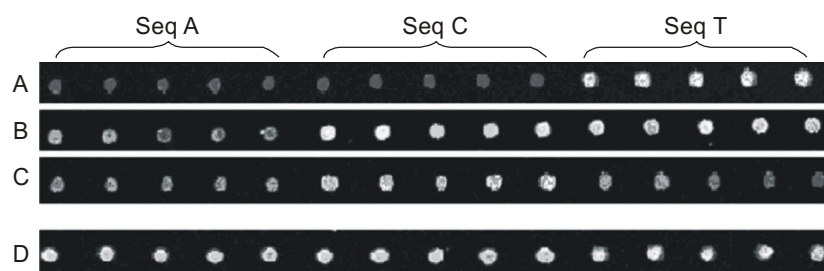
The signal ratios of different probes and the detection results

Figure	Ratios of SeqT/SeqC	Ratios of SeqT/SeqA	Ratios of SeqC/SeqA	Detection results	Genotype
4(A)	13.54	14.24	1.05	Successful	TT
4(B)	1.29	5.08	3.93	Successful	TC
4(C)	0.18	0.94	5.31	Successful	CC
4(D)	1.35	1.33	0.99	Failed	N/A

Figure 4. The hybridization results of the fluorescently labeled PCR products of primers 1 and 2. Seq T, C and A indicated on the figure corresponding to those sequences in Table 2, each oligonucleotide probe was printed in quintuplicate. (A), (B) and (C) were the results of the hybridization with the different PCR fragment of the *CYP1A1* gene, genotyping of TT, TC and CC respectively. (D) was an abnormal result of the hybridization with the PCR of TT genotype. The signal ratios of different probes and the detection results from (A), (B), (C) and (D) are listed in Table. The order of signal intensity represented by pseudo-colors from high to low is white > red > green > blue.

concluded that it is heterozygous if merely judged by the signal ratio of the Seq T/Seq C, about 1.35 (Table in Figure 4). Compared to the signal intensity of the negative control probe (SeqA), SeqC acquired an almost equal signal (the ratio of SeqC/SeqA being 0.99) that might be due to nonspecific hybridization. Therefore, without the control probe, the wrong conclusion is inevitable and we cannot even judge whether the result is reliable or not. In order to supervise the hybridization process, designing an additional probe as a negative control is recommended. When at least one of two ratios of test probes to negative control probe is greater than 1.8, the hybridization experiment is successful. Otherwise, the experiment fails due to non-specific hybridization to the mismatch probe.

Discussion

The probe density was found to be a controlling factor for the efficiency of target capture as well as for the kinetics of the target/probe hybridization (Southern *et al.,* 1999). A low surface coverage will yield a correspondingly low hybridization signal, and thus decrease the hybridization rate. Accordingly, the intensity of the hybridization signal can differ substantially from one spot to another because of variability of the density of immobile probes on the slide (Southern *et al.,* 1999; Huang *et al.,* 2001; Peterson *et al.,* 2001; Guo *et al.,* 2002). The variance of probe density is derived from many factors, including the properties of the modified slides, spotting solution, printing protocol, post-spotting procedures and the quality of probes. However, it's difficult to accurately control the spotting process, the immobilization efficiency or other factors except for adjusting the initial spotting concentration. Besides the strict control on the procedure of array fabrication, the amount of probe for spotting must be more than the maximum loading capacity of modified slides in order to ensure homogeneous densities of the different probes on the same slide. Under our experiment conditions, the initial concentration of 10 μM for spotting has been proven to be sufficient with aldehyde slides.

Our results in general correlate with previous experimental findings involving the differences in stability of oligo-DNA duplexes with different mismatched bases at the same position. The findings promise to improve the design of oligonucleotide probes for SNP/ mutation detection and save cost on unreasonable design. Generally, there are two alleles presented at one single nucleotide polymorphism locus and two corresponding probes are designed to detect its genotype through comparing signal intensity between them after hybridization. In adopting a two probe design strategy there is the likelihood of achieving high false results as factors affecting the signal in-

tensity of probe spots are complicated and unpredictable. It is likely that the signal ratios of one probe to another vary significantly under abnormal experimental conditions. The reasonable strategy is to set a negative control probe that has a different base at the mutation site compared to the two original alleles. Considering the identity of the bases, selecting the proper mutated nucleotide in the control probe is essential for discriminating the wild-type homozygous, mutation homozygous, and heterozygous in SNP detection. If it is still difficult to discriminate even when adding a control probe, an additional control probe may be used in order to ensure the accuracy of microarray detection.

Acknowledgement

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