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Attachment of oligonucleotide probes to poly carbodiimide-coated glass for microarray applications

Naoki Kimura*, Ryuichi Oda, Yoshiaki Inaki[1] and Osamu Suzuki

Research and Development Center, Nisshinbo Industries Inc., 1-2-3 Ohnodai, Midori-ku, Chiba-shi, Chiba 267-0056, Japan and [1]Material and Life Science, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

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ABSTRACT

Oligonucleotide-based DNA microarrays are becoming increasingly useful tools for the analysis of gene expression and single nucleotide polymorphisms (SNPs). Here, we present a method that permits the manufacture of microarrays from non-modified oligonucleotides on a poly carbodiimide-coated glass surface by UV-irradiation. The use of UV-irradiation facilitates an increase in the level of signal intensity, but it does not affect signal discrimination by the oligonucleotides immobilized on the surface. The signal intensity obtained for an array fabricated using non-modified oligonucleotides with UV-irradiation is ~7-fold greater than that without UV-irradiation. The detection of SNPs was tested to ascertain whether this technique could discriminate specific hybridization signals without causing significant UV-irradiation-induced damage to the immobilized oligonucleotides. We found that this immobilization method provides greater hybridization signals and a better match/mismatch ratio of SNPs than do the established aminosilane techniques. Application of this technology to manufacturing DNA microarrays for sequence analysis is discussed.

INTRODUCTION

In the past few years, DNA microarray technology has become a fundamental tool for the detection and analysis of nucleic acid sequences. Major applications of this technique include studying gene expression profiles and the detection of single nucleotide polymorphisms (SNPs). DNA microarray products that utilize optical, electrochemical and mechanical detection methods have been developed (1–10).

Because of the favorable optical characteristics, DNA chips are usually fabricated using a glass slide as the support medium. Oligonucleotides or PCR products are spotted onto an activated glass surface, such as one coated with aminosilane, poly-lysine, aldehyde, epoxy or active esters (3–6). Alternative methods involving either *in situ* oligonucleotide synthesis (1,2) or a pseudo-three-dimensional structure using a dendrimeric linker system (8) have been also developed. The performance of a surface in terms of signal intensity and signal-to-noise ratio is directly influenced by the amount of DNA probe attached to the surface and by the accessibility of the labeled target to the probe. An ideal support should have a surface chemistry that allows stable covalent binding of DNA at high capacity. Furthermore, the DNA must be accessible to the probe so as to allow discrimination between similar target sequences.

Oligonucleotide microarrays are potentially more cost-efficient than cDNA microarrays. However, preparation of the oligonucleotides is a major factor in the production of a DNA microarray in terms of both time and cost. In most cases where slide surfaces coated with aldehyde, active esters or gold are used, the oligonucleotides are generally modified by the introduction of both a suitable moiety, such as an amino or thiol group, to significantly increase their binding capacity to the array, and a spacer unit to avoid steric hindrance (5–8). Although the quality, accuracy and reliability of these microarrays have been demonstrated, modification with a linker group requires additional procedures, thereby increasing the cost of fabrication. The yield of modified oligonucleotide is generally low and purification by HPLC is usually required. In addition, the amino group has the limitation of being pH sensitive in its reaction. Amines are protonated at low to neutral pH, decreasing the reaction rate. The reaction of amines with active esters is fast at high pH values, but the decay of the active esters is also accelerated. In contrast, the fabrication of aminosilane arrays utilizing non-modified oligonucleotides, which provide gene expression profiles comparable to those utilizing PCR products, has also been reported (10). Thus, the fabrication of an array using non-modified oligonucleotides that does not compromise the quality of the DNA chip would avoid the extra steps and result in a substantial cost saving (10).

Carbodiimides are reactive functional groups used in DNA modification (11). Over the past few years our laboratory has developed the production of poly carbodiimide for attachment studies between carbodiimide groups and non-modified DNA (12). However, short non-modified oligonucleotides show low reactivity with carbodiimide groups under standard

*To whom correspondence should be addressed. Tel: +81 43 205 0794; Fax: +81 43 205 0843; Email: n-kimu@nisshinbo.co.jp

Table 1. Sequence of oligonucleotides used in this study.

	Gene target	Forward primer sequence	Reverse primer sequence	GenBank ID	Mutation point
1	CCKAR–1	gtactggggaagcctctgtg	caccttcaccccgccccac	306595	G(–128)T
2	β3AR	gcgctggcggtgctggc	gcggccagcgaagtcacg	312396	T192C
3	GNB3	ctgatctgcttctcccacgag	gaatagtaggcggccactgag	20257501	C825T
4	PPARγ-2	cacaggtgcaatcaaagtggag	cttattgtagagctgagtcttctc	23953882	C345A
5	PPARγ-1	gttatgggtgaaactctgggag	tatgtttgcagacagtgtatcagtg	23953882	C36G
6	phage λ DNA	tcgccccgctgtttttgatga	catcgtcgcgccggtagtcat	215104	

Non-fluorescently-labeled oligonucleotides and 5′-fluorescently-labeled oligonucleotides were prepared as PCR primers (same sequence). Non fluorescently-labeled forward primer of phage lambda DNA was also employed as probe DNA for immobilization on the slide.

immobilization conditions (11,12), giving weak hybridization signals (signal-to-noise ratio).

In this paper we describe a new method for attaching oligonucleotides to a solid support for DNA microarray applications. Non-modified oligonucleotides are efficiently immobilized onto a poly carbodiimide-coated glass slide by UV-irradiation. No toxic reagents are required. This technique offers a means of producing oligonucleotide microarrays that is both quicker and cheaper than current methodologies. The quality of these arrays is not compromised as judged by their ability to discriminate SNPs of oligonucleotides and PCR products upon hybridization. Furthermore, the performance of this technology is also compared with commercially available aminosilane slides utilizing the same non-modified oligonucleotides.

MATERIALS AND METHODS

Synthesis of oligonucleotides

Modified oligonucleotides were synthesized on an ABI 3900 DNA synthesizer at a 0.2 μmol scale using the standard phosphoramidite method. Oligonucleotides modified with a 5′-amino group and a six-carbon spacer and labeled with 5′-hexachlorofluorescein (HEX) were purified by reverse-phase HPLC using a standard procedure and dried *in vacuo*. HPLC-purified 5′-Cy5-labeled oligonucleotides were purchased from Yamahisa Kasei Inc. Non-modified oligonucleotides were also synthesized on an ABI3900 DNA synthesizer at a 0.2 μmol scale using standard procedures. The non-modified oligonucleotides were then purified on a reverse-phase cartridge following a standard procedure and dried *in vacuo*.

Fabrication of poly-carbodiimide-coated arrays

A poly carbodiimide-coated slide (Carbo Station™, Nisshinbo Industries Inc.) platform consists of a glass slide that has been produced by coating microscope slides with poly carbodiimide. The average degree of polymerization of the polymer is 10, including the alkyl spacer group and a carbodiimide group, which provides the attachment site for thymine bases of oligonucleotides.

Manual spotting procedure. Either non-modified oligonucleotides or mixtures of the non-modified oligonucleotide and the HEX-labeled oligonucletoide were dissolved in 3× SSC at 10 pmol/μl concentration and spotted in triplicate manually with a 2 μl Pipetteman on the slide as 0.5 μl droplets, ~1 mm in diameter and 2 mm center-to-center.

Spotting procedure using a microarray robot. A Pixsys DNA microarray spotter (Cartesian technologies) with a SMP10B pin (TeleChem International Inc.) was also used to print arrays on poly carbodiimide-coated glass slides. Either non-modified oligonucleotides or mixtures of the non-modified oligonucleotide and the HEX-labeled oligonucletoide in 3× SSC (total volume 20 μl) were used to print arrays, ~250 μm in diameter and 600 μm center-to-center. The humidity was maintained around 70% during printing. Oligonucleotides were printed from 96-well plates (NUNC) at the desired concentration.

Immobilization procedure. After printing, the arrays were irradiated (total 0–0.6 J/cm^2) using a UV Stratalinker 2400 (Stratagene) with a central irradiation wavelength of 254 nm, treated with a blocking buffer [3% BSA, 0.2 M NaCl, 0.1 M Tris–HCl (pH 7.5) and 0.05% Triton-X100], washed with TE buffer (pH 7.2) for 5 min, and dried for storage. UV-irradiation was carried out within 5 min. All procedures were performed at room temperature.

Fabrication of the aminosilanized arrays

GAPS2-coated slides from Coring and Super Amine slides from TeleChem International were used in this study. A Pixsys DNA microarray spotter with the same SMP10B pin was also used to print arrays at a concentration of 50 pmol/μl for the non-modified oligonucleotides in 3× SSC as described above. After printing, the arrays were UV-irradiated (i.e. 0.15 J/cm^2 for GAPS2 coated slides and 0.45 J/cm^2 for Super Amine slides), washed and treated with the blocking solution according to the manufacturer's recommended procedure or a previously reported method (10). Prior to fabricating the aminosilane arrays, we compared immobilization procedures (i.e. UV-irradiation *versus* incubation procedure) utilizing non-modified and NH_2-modified oligonucleotides (4,10). However, the incubation procedure resulted in a decrease in sensitivity under the conditions used in this study. Furthermore, we confirmed that the hybridization signals for the UV-irradiated procedure became saturated at a non-modified oligonucleotide concentration of 50 pmol/μl.

PCR amplification

The sequence of each primer is shown in Table 1.

Amplification of human DNA for the PCR-based genotype study. PCR was performed on a mixture of 100 ng of human genome DNA (Roche), 50 pmol of non-fluorescently-labeled

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forward primer, 50 pmol of non-fluorescently-labeled reverse primer and 25 µl of HotStarTaq Master Mix kit (Qiagen) in a total volume of 50 µl. Amplification consisted of 39 cycles, with denaturation at 95°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The initial denaturation was at 95°C for 15 min. Finally, the PCR mix was incubated at 72°C for 3 min.

Amplification of bacteriophage lambda DNA for reuse of the array. The PCR mix included 5 ng of phage lambda DNA (TAKARA), 20 mM Tris–HCl (pH 8.0 at room temperature), 100 mM KCl, 2.5 mM $MgCl_2$, 5 pmol of non-fluorescently-labeled forward primer, 5 pmol of non-fluorescently-labeled reverse primer, 2.5 mM each deoxynucleoside triphosphate and 2.5 U of r-Taq (TAKARA) in a total volume of 50 µl. Amplification consisted of 40 cycles, with denaturation at 95°C for 1 min, annealing at 60°C for 30 s and extension at 72°C for 30 s. The initial denaturation was at 95°C for 5 min. Finally, the PCR mix was incubated at 72°C for 3 min.

Purification and cloning of the PCR products. All PCR products were purified using the Qiaquick PCR purification kit (Qiagen), and cloned into the pGEM-Teasy vector (Promega) following the manufacturer's protocol. The plasmids were amplified in *Escherichia coli* JM109 (Toyobo). The products were purified by a Plasmid purification kit (Qiagen). The template human DNA allowed the amplification of certain genes in a heterogeneous state. Four plasmids were prepared for each PCR product to generate a heterogeneous mix of constructs. For example, a ADRβ3-amplified product was generated as a mixture of a wild type and a mutated gene (T192C, see Table 1) during the PCR.

Generation of fluorescently-labeled PCR products. For the generation of complementary hybridization targets, PCR reactions were performed using fluorescently-labeled forward and reverse primers with plasmid DNA as template. The PCR temperature cycle was the same as that used above. The PCR products were applied to a 5% acrylamide gel and visualized by staining with ethidium bromide. The size of each PCR product was from 70–167 bases for human DNA and 300 bp for bacteriophage lambda DNA. The sequences of the products were confirmed by ABI 310 genetic analyzer.

Hybridization

Hybridizations were performed as follows.

Oligonucleotide targets. Two different fluorescently labeled oligonucleotides (3.3 ng of each target) were dissolved in distilled water and denatured for 1 min at 95°C. Uni-Hybri solution (TeleChem International Inc.) was then added (80% v/v Uni-Hybri solution; total volume 20 µl), and the hybridization solution was applied by capillary action between a slide and cover slip. The slide was incubated for 0.5 h at 37°C in a closed hybridization cassette. Subsequently, the arrays were washed at room temperature in 2× SSC –0.1% SDS for 5 min, rinsed briefly in 2× SSC and dried by centrifugation at 500 r.p.m. (CS-15 Centrifuge, BECKMAN) for 2 min.

PCR product targets. Each fluorescently labeled PCR product (46–198 ng) was dissolved in distilled water and denatured for 1 min at 95°C. Uni-Hybri solution was also added (80% v/v Uni-Hybri solution; total volume 20 µl) and the hybridization solution was applied by capillary action between a slide and cover slip. The slide was incubated for 2 h at 42°C in a closed hybridization cassette. Subsequently, the arrays were washed at room temperature in 2× SSC –0.1% SDS for 5 min, followed by 5 min in 0.2× SSC –0.1% SDS at 40°C, rinsed briefly in 0.5× SSC and dried by centrifugation as described above.

Signal detection and data analysis

The arrays were imaged on a Scan Array 4000 unit (Packard Biochip) with 10 µm resolution. A Cy3 or Cy5 optical filter was used during imaging of the arrays. The laser power and photomultiplier tube voltage (PMT) were always set to 100 and 80% for the Cy5 channel, respectively; the laser power was set to 100% varying PMT settings to obtain optimum signal intensities for the Cy3 channel. Analysis of the intensity of the original 16-bit tiff images from either a Cy3 or a Cy5 channel was performed using Quant Array software (Packard Biochip), and graphs were generated in Microsoft Excel. Unless stated otherwise, the average signal values were taken from three spots on three slides processed in parallel. The data were further validated by results from more than five hybridization experiments.

UV-irradiation applied to oligonucleotides in the absence of poly-carbodiimide

Non-modified oligonucleotide probe was dissolved in 3× SSC at the concentration of 100 pmol/µl. The solution (50 µl) was transferred to a petri dish, and exposed to 254 nm light (ranging from 0 to 1.2 J/cm^2) using a UV Stratalinker 2400. The UV-irradiated oligonucleotides were diluted 20-fold and the absorbance at 260 and 320 nm measured in a 10 µl fused quartz cell using a spectrophotometer (Gene Spec III, Naka Instrument Co., Ltd).

Stripping procedure

After hybridization with Cy5-labeled target (phage lambda DNA fragment, see Table 1), the slides were immersed in distilled water at 95°C for 5 min. They were then rinsed in distilled water and dried for storage at room temperature.

RESULTS

Effect of UV-irradiation on the poly carbodiimide-coated glass surface

The amount of probe attached to the solid support and the accessibility of target DNA to the probe are critical factors in microarray analysis, and they determine both the sensitivity and dynamic range of measurement. To investigate how UV-irradiation during immobilization on the poly carbodiimide-coated glass surface affects hybridization efficiency and discriminative ability, we used eight different non-modified oligonucleotide (10- and 12mer) probes (Fig. 1). Each non-modified oligonucleotide probe was designed to be centrally located around each SNP site in the target sequences (13,14). The presence of multiple and single thymine bases within the

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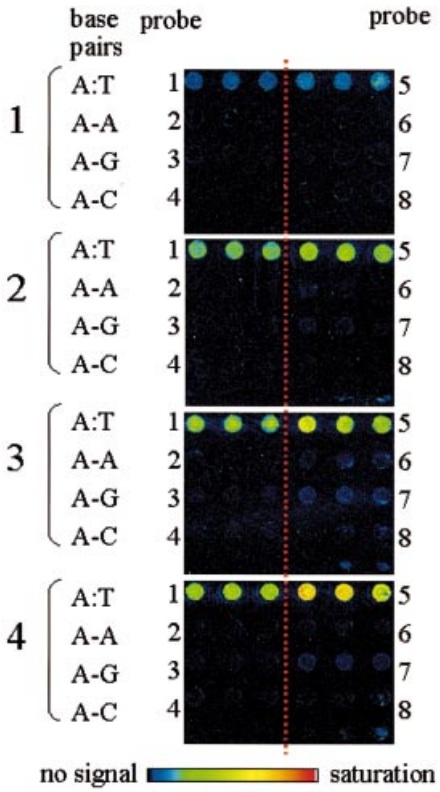

Figure 1. Signal intensities produced upon hybridization of HEX-labeled oligonucleotide targets to eight different spotted oligonucleotide probes at 0–0.6 J/cm^2 of UV-irradiation. Spots made with each non-modified oligonucleotide probe were present in triplicate. Non-modified oligonucleotide probe sequences were as follows: 5′-GAGTAT**X**ATGAG-3′, X is thymine (probe 1), adenine (probe 2), guanine (probe 3) and cytosine (probe 4), respectively; 5′-CGCAT**X**ACGC-3′, X is thymine (probe 5), adenine (probe 6), guanine (probe 7) and cytosine (probe 8), respectively. Immobilization was performed using (1) 0, (2) 0.15, (3) 0.3 and (4) 0.6 J/cm^2 of UV-irradiation. The slides were hybridized in parallel with two different HEX-labeled oligonucleotides (12- and 10mer), and washed briefly. Oligonucleotide target sequences were as follows: 3′-CTCATA**A**TACTC-5′ and 3′-GCGTA**A**TGCG-5′. Positions of nucleotide substitutions are underlined. All arrays were scanned with same laser power (100%) and PMT setting (85%).

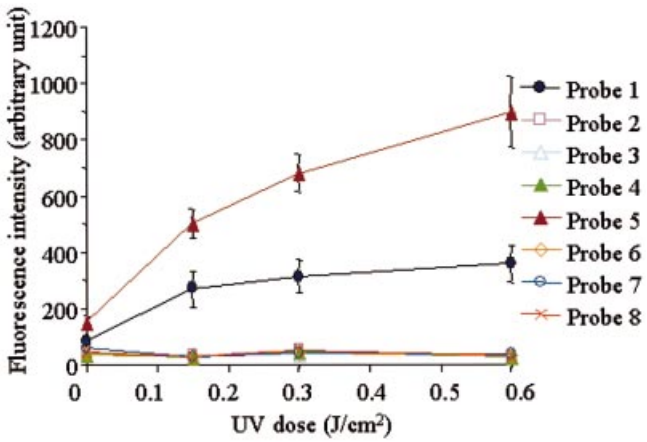

Figure 2. Effect of UV-irradiation on hybridization efficiency. The mean signal intensities produced in the experiments shown in Figure 1 are plotted versus UV dose. The error bars indicate standard deviation.

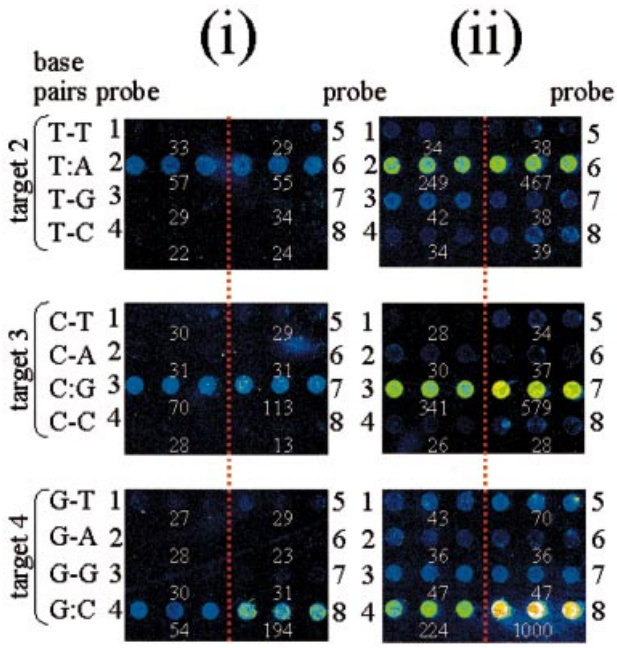

Figure 3. Hybridization of the same array of probes to six HEX-labeled oligonucletoide targets. Four 12- and four 10mer non-modified oligonucleotides, as shown in Figure 1, were spotted on the slides. Immobilization was performed using (i) 0 and (ii) 0.6 J/cm^2 of UV-irradiation. Non-modified oligonucleotide probe sequences are shown in Figure 1. The slides were hybridized in parallel with six different HEX-labeled oligonucleotides (12- and 10mer), and washed briefly. Oligonucleotide target sequences were as follows: target 2: 3′-CTCATA**T**TACTC-5′ and 3′-GCGTA**T**TGCG-5′; target 3: 3′-CTCATA**C**TACTC-5′ and 3′-GCGTA**C**TGCG-5′; target 4: 3′-CTCATA**G**TACTC-5′ and 3′-GCGTA**G**TGCG-5′. Positions of nucleotide substitutions are underlined. At the bottom of the corresponding spots is given the mean value of fluorescent signal. All arrays were scanned with same laser power (100%) and PMT setting (85%).

different probes makes them particularly suitable for addressing the potential problems associated with UV-irradiation. The non-modified oligonucleotide probes are most likely attached to the poly carbodiimide via thymine base(s) by UV-irradiation. The non-modified oligonucleotide probes were dissolved in 3× SSC at a concentration of 10 pmol/μl, and applied to the glass slide in triplicate manually.

In hybridization experiments, a mixture of two different HEX-labeled oligonucleotides, which were complementary to Probes 1 and 5 respectively, was used as target DNA. Figure 1 shows a typical image of fluorescence signal intensities from these experiments. Irrespective of UV dose, hybridization was specific to the complementary probe DNA. Signal intensities increased with increasing UV dose, whereas non-specific signals were at background levels. Using 3× SSC solution as printing buffer and UV dose up to 0.6 J/cm^2, the signal intensity was ~4.5-fold (probe 1) and 5.9-fold (probe 5) greater for the UV-irradiated array than for the non-irradiated control (Fig. 2). Interestingly, we did not observe an obvious increase in non-specific hybridization signals (i.e. probes 2–4 and 6–8) when the UV dose was elevated to 0.6 J/cm^2. Further investigation was performed using six different oligonucleotide targets, which were complementary to probes 2–4 and 6–8, respectively. Figure 3 illustrates a similar trend with these additional targets. The signal intensities with targets 2–4 were

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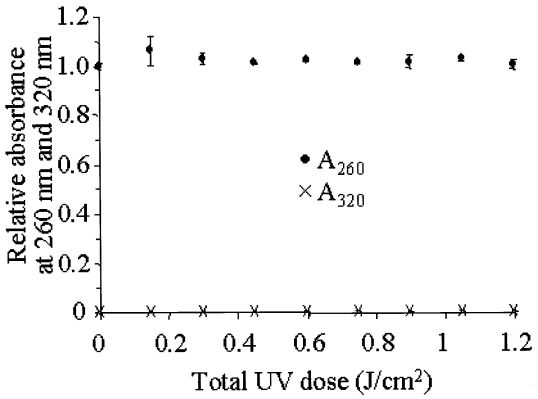

Figure 4. Relative absorbance in UV-irradiated oligonucleotide (probe 1) ranging from 0 to 1.2 J/cm^2. The doses are plotted as the sum of doses at each absorbance (260 and 320 nm).

~7.1-fold greater for the UV-irradiated array than for the corresponding non-irradiated controls, with no obvious non-specific signals. We also tested hybridization specificity using the targets that bear a double base mutation at positions in the middle of the sequences. As expected, the observed hybridization signals on the UV-irradiated array showed ~16.7-fold lower intensity than that of the complementary strand (data not shown). Because the non-modified oligonucleotide probes are most likely attached to the poly carbodiimide via thymine base(s), one might envisage that the A:T base pairs are inhibited from forming during hybridization. However, these results show that the carbodiimide chemistry can discriminate a SNP site of each target (A:T match versus T-T, C-T and G-T mismatches, see Figs 1 and 3), as well as other mismatch sites (e.g. T:A match versus T-T, T-G and T-C mismatches, see target 2 in Fig. 3), in the presence of single or multiple thymine bases in probes.

To estimate the immobilized oligonucleotide surface density, HEX-labeled oligonucleotides (probes 1 and 5) were spotted in separate positions on the slide and immobilized by UV-irradiation (0 or 0.6 J/cm^2). Prior to printing, 5′-HEX-labeled probe 1 or 5 (same sequence with probe 1 or 5 as shown in Fig. 1) was mixed with non-modified probe 1 or 5 (ratio of 1:100, mol/mol), respectively, to prevent optical quenching. To give corresponding hybridization signals (UV dose 0.6 J/cm^2, see Figs 1 and 2), 3.0-fold for probe 1 and 3.4-fold for probe 5 greater oligonucleotide surface densities were observed as compared to non-UV-irradiated conditions at the same oligonucleotide concentration. Thus, the weaker hybridization signal of the non-UV-irradiated spot might be due to insufficient immobilized probe. These results indicate that the reaction between the oligonucleotide and poly carbodiimide on the glass surface is facilitated by UV-irradiation, and that hybridization involving an immobilized thymine base is only slightly impaired.

Verification of UV-induced oligonucleotides

We performed further investigations to confirm whether UV-irradiation promoted the formation of dimers that might enhance destabilization of probe/target complex during hybridization (13–17). Dimer formation can be monitored by measuring absorption changes at 260 and/or 320 nm (13–17). Probe 1 was dissolved in 3× SSC and transferred to a petri dish and UV-irradiation was performed in the absence of poly carbodiimide (0–1.2 J/cm^2). We measured absorption of the UV-irradiated oligonucleotide at 260 and 320 nm. Figure 4 shows that no decrease of absorption at 260 nm was observed during UV-irradiation up to 1.2 J/cm^2, indicating that the *cis-syn* cyclobutane was not produced. Moreover, no increase of absorption at 320 nm was observed, indicating that the (6–4) adduct was not produced. Therefore, in contrast to previously reported methods (13–17), we could not detect UV-induced dimer formation under the conditions used here. The absence of dimers may be due to either an insufficient UV dose to promote the reaction, or the absence of a sensitizer such as acetophenone that enhances dimer formation from the triplet state during UV-irradiation. A similar trend was observed with other UV-irradiated probes. The results indicate that UV-irradiated oligonucleotides under these conditions are suitable for DNA microarray application.

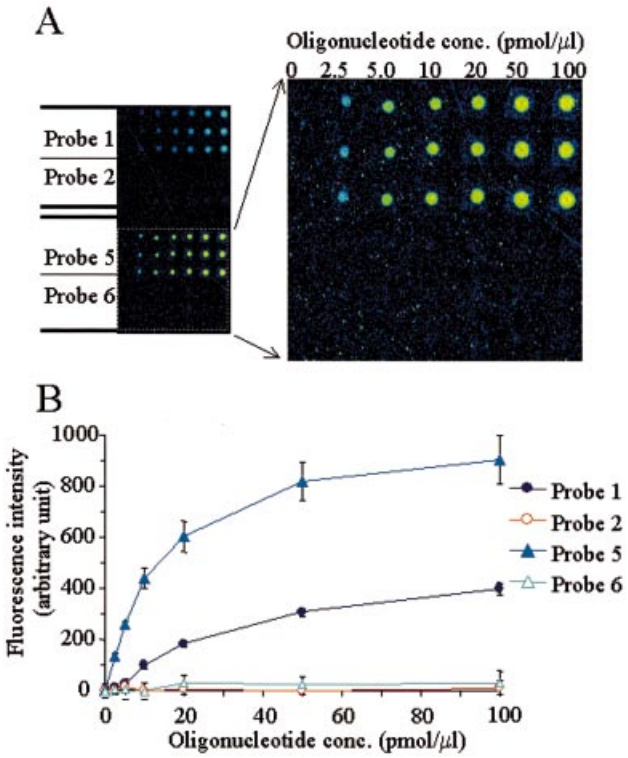

Figure 5. Effect of probe concentration on hybridization efficiency. Non-modified oligonucleotide probes (probes 1–2 and 5–6) were spotted on the slides. Immobilization was performed using 0.6 J/cm^2 of UV-irradiation. The slides were hybridized in parallel with the mixture of HEX-labeled oligonucleotide targets, and washed briefly. Non-modified oligonucleotide sequences are shown in Figure 1. (**A**) Image capture from hybridization of arrays bearing DNA probes spotted at increasing concentrations. (**B**) The mean signal intensities produced in the experiments shown in (A) are plotted versus non-modified oligonucleotide concentration of the spotted oligonucleotide. The error bars indicate standard deviation. All arrays were scanned with the same laser power (100%) and PMT setting (85%).

Hybridization capacity

High surface densities and a strong hybridization signal might not necessarily be related, because steric interference between immobilized probes could hinder access to DNA target and

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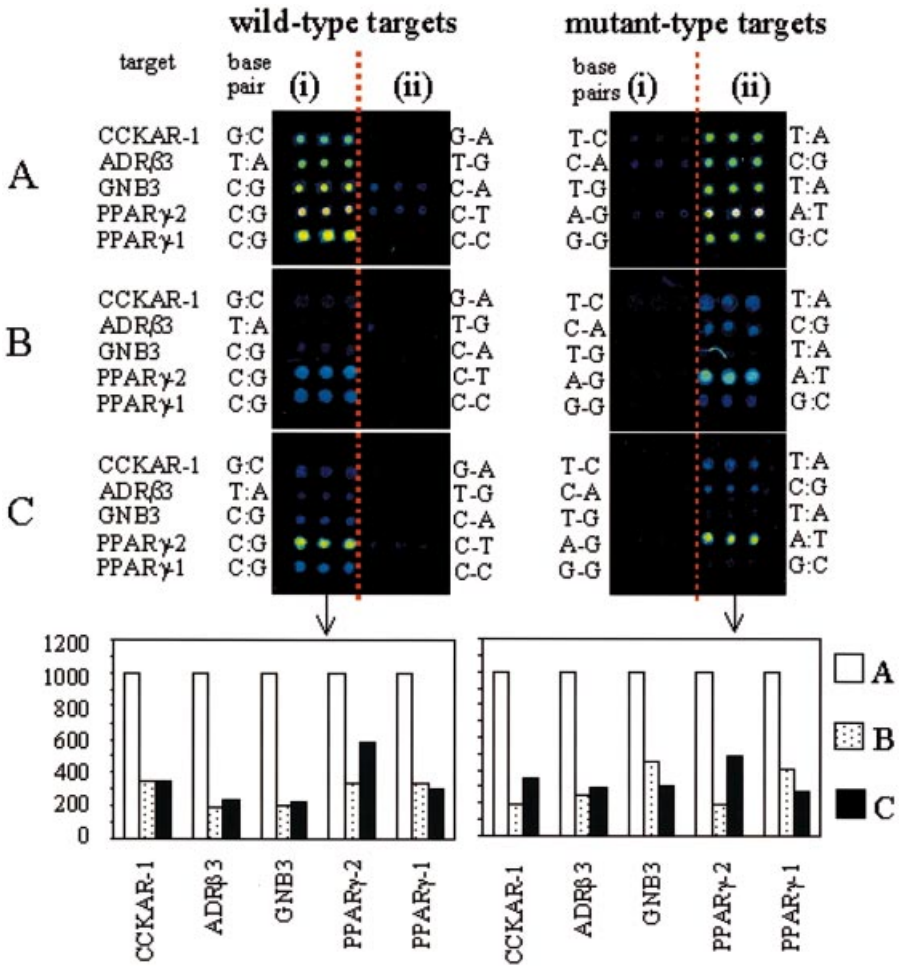

Figure 6. Hybridization of the same array of ten probes to HEX-labeled PCR products from wild-type or mutant genes. (**A**) Non-modified oligonucleotide probes were dissolved in 3× SSC at a concentration of 50 pmol/μl and spotted on the slides in triplicate. After immobilization with 0.6 J/cm^2 of UV-irradiation, the slides were treated with buffers and dried. (i) Wild-type hybridization of HEX-labeled PCR products, (ii) mutant hybridization of HEX-labeled PCR products hybridized to the arrays. Hybridizations were performed using a mixture of five wild-type targets, and a mixture of five mutant-type targets respectively (see Table 1). HEX-labeled PCR products also hybridized to (**B**) the GAPS2 array and (**C**) the Super amine array fabricated with the same non-modified oligonucleotides are shown. The bar graphs represent the mean signal intensities of specific hybridization signals among different arrays. The signal intensity was calculated by dividing the mean signal intensity of the spot areas on (B) GAPS2 [(C) Super amine] arrays by the mean signal in between spots on (A) the poly carbodiimide-coated arrays. The average signal values were taken from three spots on six slides processed in parallel. [Note: the maximum mean signal intensity of each specific hybridization among different arrays was normalized to 1000 (no units) representing the relative intensity of the corresponding DNA.] All arrays were scanned with the same laser power (100%) and PMT setting (80%).

reduce the hybridization efficiency (4,5,9). Therefore, we studied the immobilization capacity of poly carbodiimide-coated slides to obtain optimal hybridization intensity (signal-background) (Fig. 5). To this end, probes 1 and 5 were diluted with 3× SSC to concentrations of 100, 50, 20, 10, 5 and 2.5 pmol/μl, and applied to the glass slide in triplicate using a microarray robot (see Materials and Methods). As a negative control, probes 2 and 6 were also dissolved in 3× SSC at the same concentration and spotted onto the same slide. Spotting solution without oligonucleotide was also deposited. Immobilization was performed by UV-irradiation (0.6 J/cm^2), as shown in Figures 1–3.

In hybridization experiments, HEX-labeled oligonucleotides were also used as target DNA (Fig. 1). As expected, signal intensities increased with increasing non-modified oligonucleotide concentrations, whereas non-specific signals were minimal across all non-modified oligonucleotide concentrations (Fig. 5A). Correspondingly, Figure 5B shows that the hybridization capacity of the glass surface is nearly saturated at a DNA concentration of 50 pmol/μl for both probes 1 and 5 at 0.6 J/cm^2. For all these processes, one might expect a reduction in the amount of bound DNA at higher concentrations because of steric reasons. However, within the range analyzed, no such effect was observed.

Discrimination of mismatch for biological application

DNA microarray technology is widely used to detect single mutations in a given gene (SNPs) (4,5). Although techniques to identify SNPs are available, there remains a requirement for developing an inexpensive, high throughput method to screen for point mutations, especially in terms of medical diagnostics. Therefore, we examined the potential application of our carbodiimide chemistry to further develop the microarray technology. The discrimination of single nucleotide mis-

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Table 2. Comparative study of the M/MM ratios determined on different types of slide

Gene target	Wild-type hybridization Poly carbodiimide	GAPS2	Super amine
CCKAR–1	7.2	2.3	2.0
β3AR	8.5	1.4	1.5
GNB3	6.9	2.4	2.7
PPARγ-2	13.9	6.3	9.8
PPARγ-1	20.1	5.3	6.5
	Mutant-type hybridization		
CCKAR–1	11.3	1.3	4.1
β3AR	4.9	2.9	4.4
GNB3	8.9	1.9	1.7
PPARγ-2	17.2	6.8	20.3
PPARγ-1	11.1	1.9	1.6

Shown are the mean M/MM ratios of the hybridization signals of wild-type and mutant sequences produced in the experiments described in Figure 6. The mean M/MM ratios were derived from three spots on six slides processed in parallel.

matches derived from a PCR-based oligonucleotide typing system was tested. We isolated the human genes of interest by PCR using combinations of primers (Table 1), and cloned the amplified products into a plasmid. Clones of the wild-type and mutant products were easily generated and isolated. The size of both wild-type and mutant PCR product fragments (CCKAR-1, β3AR, GNB3, PPARγ-2 and PPARγ-1) were 167, 110, 78, 70 and 76 base pairs, respectively (18–22). Each PCR product contains one SNP site (Table 1). The immobilized oligonucleotide probes (15mers) were designed to be centrally located around each SNP site in the target sequences. With these two short probes arrayed on the glass slide, the hybridization to each target generates two match/mismatch (M/MM) combinations. In total, 10 different non-modified oligonucleotide probes for detecting 10 corresponding target sequences were spotted and immobilized on the same array. Each non-modified oligonucleotide probe (50 pmol/μl in 3× SSC) was printed and immobilized by UV-irradiation (0.6 J/cm^2) as shown in Figure 5.

Hybridization results are presented in Figure 6A. As expected, in all cases a difference in hybridization signal is evident between the fully matched probe and one containing a single mismatch with a hybridized target. The M/MM ratio of each signal was calculated by dividing the mean signal intensity of the three spot areas of the wild-type (mutant) signal by the mean of the mutant (wild-type) signal in between spots (Table 2). Quantification analysis shows that the relative fluorescence intensity between wild-type and mutant specific oligonucleotide probes on each array differs by 4.9–20.1-fold, thereby allowing single M/MM discrimination. The array is not optimized in terms of hybridization properties, but performance was consistent with the expected properties of DNA duplexes in solution. We found that UV-irradiation of non-modified oligonucleotides spotted onto poly carbodiimide-coated glass slides during immobilization allowed single M/MM discrimination.

Comparison of hybridization efficiency with different attachment chemistries

The performance of different attachment chemistries (i.e. most widely used aminosilane slides) was also assessed by testing the discrimination of SNPs derived from a PCR-based oligonucleotide typing system, as shown in Figure 6A. A single SMP10B pin was used throughout the study to avoid differences between pins. Furthermore, any inconsistency in the poly carbodiimide-coated array resulting from different print sessions or from variations in day-to-day hybridization and wash procedures were eliminated. We compared our method with aminosilane methods utilizing the same non-modified oligonucleotide probes at the same concentration upon hybridization.

In our hands, however, weaker hybridization signals were obtained with the aminosilane slides (Fig. 6B and C) using hybridization and scanning conditions (laser power: 100%, PMT 80%) identical to those used in our methodology. Quantification also revealed relatively lower hybridization signals (~5.3-fold) in comparison to those obtained using our method. Furthermore, we observed relatively poor M/MM ratios between wild-type-specific and mutant-specific non-modified oligonucleotide probes on the aminosilane arrays, probably due to the overall lower fluorescent intensities, although the aminosilane arrays could still discriminate between the wild-type and mutant genes (Table 2).

Thus, we find that carbodiimide chemistry provides a better substrate for the immobilization of short non-modified oligonucleotides and hybridization than do other slides.

Tight binding of nucleic acids on the slide surface

Finally, we investigated whether the slide (immobilized DNA on the slide by UV-irradiation) was reusable. The reuse of a DNA microarray could be an advantage in genetic studies because of the significant cost savings. To determine the amount of DNA retained on the slide surface after harsh treatment, we immobilized a mixture of HEX-labeled and non-modified oligonucleotide (forward primer of bacteriophage lambda DNA; see Table 1, ratio 1:100, mol/mol) on the poly carbodiimide-coated slide surface. The oligonucleotide sequences were complementary to bacteriophage lambda DNA. After immobilization (0.6 J/cm^2 UV-irradiation), blocking and washing, the slides were boiled with distilled water for 5 min to remove any unbound DNA. We found 78.5% of the DNA was retained on the surface and that a second wash retained 91% of the DNA. This result suggests that the remaining nucleic acids were tightly linked to the slide by covalent bonding. The experiment led us to think that the slides could be used multiple times by repeated stripping.

After an initial hybridization with the Cy5-labeled lambda DNA fragment, the arrayed diazotized slides were stripped and re-hybridized with the same Cy5-labeled lambda target four successive times. Stripping resulted in the complete removal of all signal (data not shown). Images of the spots resulting from each hybridization and quantification of the mean intensities yielded the results shown in Figure 7. We observed significant signal loss after each round of hybridization and stripping presumably due to the harsh stripping conditions (95°C for 5 min). However, whereas the signal intensity decreased with each successive hybridization, the spots on the poly carbodiimide-coated slide clearly remained detectable and proportionately accurate, reflecting the amount of specific probe immobilized. These findings confirm that the immobilization process resulted in very stable binding of the nucleic acid moiety to the glass surface, suggesting that the arrays can be reused.

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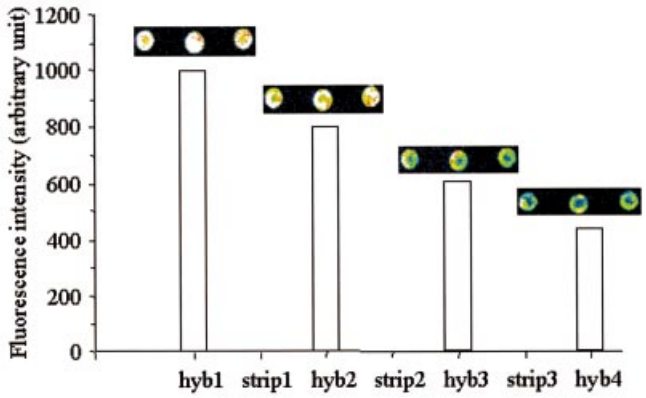

Figure 7. Effect of stripping on re-hybridization of Cy5 labeled target to the immobilized oligonucleotide without modification on the poly carbodiimide-coated slide surface. Scanned spots and quantitative analysis of spots over hybridizations 1–4 (where 1 is the initial hybridization, and 2–4 are sequential hybridizations after repeated stripping) are shown. All arrays were scanned with the same laser power (100%) and PMT setting (80%).

DISCUSSION

In this study, we have shown that the signal intensities obtained for a poly carbodiimide-coated array fabricated using non-modified oligonucleotides with UV-irradiation are enhanced, and that this technique can discriminate specific hybridization signals without causing significant UV-irradiation-induced damage to the immobilized oligonucleotides. Although the UV-induced covalent linkage of thymine bases and amino groups on a solid surface has been reported previously (10,23) and it is well known that a carbodiimide group reacts with a thymine or guanine base in DNA (11,12), to our knowledge, this is the first report to show that UV-irradiation of spotted oligonucleotides on a poly carbodiimide-coated slide facilitates an increase in hybridization signal. The enhanced quality of the UV-immobilized DNA array is probably due to the increased amount of oligonucleotide on the slide as well as allowing probe–target interaction during hybridization. In addition, unlike the amino surface, the poly carbodiimide surface has no electrostatic interaction with the DNA, which may also enhance the quality of the array.

Furthermore, we compared our methodology against the widely used aminosilane chemistry coupled to UV-irradiation immobilization procedures by testing arrays made utilizing the same set of short non-modified oligonucleotides. However, a direct comparison of different immobilization chemistries is difficult because of the many factors involved. These include surface conditions such as loading capacity, charge, hydrophobicity and efficiency of immobilization and hybridization. Nonetheless, our results demonstrate that UV-induced binding of DNA to poly carbodiimide-coated slides generates a relatively superior quality DNA microarray as compared with the standard aminosilane methodology. Therefore, our method has advantages for DNA microarray applications not only because of the cost-effectiveness of using non-modified oligonucleotides as reported previously (10), but also because our arrays yield stronger hybridization signals and better specificity than do the widely used aminosilane slides.

In this study, we used a model system consisting of a relatively small number of distinct nucleic acid species to compare two different methodologies. Therefore, to apply and to assess the general performance of carbodiimide chemistry to gene expression analysis, we performed reverse transcriptions to produce cDNAs labeled with two different cyanine dyes from 1 to 10 μg total mouse liver or brain RNA. We then used these labeled cDNA targets to hybridize poly carbodiimide chips containing 70mer non-modified oligonucleotides attached by UV-irradiation from a set of randomly chosen 1552 mouse gene sequences provided on the website of Wang *et al*. (24). A quantifiable fluorescent signal on almost all spots could be recorded, even with the lowest amount of cDNA targets (N.Kimura, M.Akiyama and T.Tamura, submitted).

Thymine is known to give a photo-dimer upon irradiation by UV light near the λ_{max} (280 nm) of thymine (13–17). The immobilization reactivity was found to vary depending on the number of thymine bases in the DNA molecule. The action spectra (data not shown) indicated 280 nm to be the most efficient wavelength for immobilization of DNA. From these facts, the reaction depends primarily on the excited thymine bases, although the product between oligonucleotide and poly carbodiimide has not been isolated. One possible explanation is that the carbon of a carbodiimide group may react at position 6 of the thymine base in common with production of the T-T dimer (6–4) by UV-irradiation (13–15). Therefore, we cannot discount the possibility that immobilized DNA comprising frequent thymine bases on poly carbodiimide-coated slides may adversely limit oligonucleotide accessibility and flexibility to the DNA target during hybridization. Further studies are needed to investigate the possible impact of T-rich oligonucleotide probes on the immobilization process.

In conclusion, immobilization of non-modified oligonucleotides on the poly carbodiimide-coated slides by UV-irradiation led to a significant improvement in the performance of the microarrays in terms of reproducibility and accuracy of measurement. Moreover, slides can be stored for 6–12 months at room temperature before use. A fabrication process based on these findings should assist in the development of high quality arrays at an affordable price.

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REFERENCES

1. Watson,A., Mazumder,A., Stewart,M. and Sriram,S. (1998) Technology for microarray analysis of gene expression. *Curr. Opin. Biotechnol.*, **9**, 609–614.
2. Fodor,S.A., Rava,R., Huang,X.C., Pease,A.C., Holmes,C.P. and Adams,C.L. (1991) Light-directed, spatially addressable parallel chemical synthesis. *Science*, **251**, 767–773.
3. Schena,M., Shalon,D., Davis,R.W. and Brown,P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*, **270**, 467–470.
4. Guo,Z., Gatterman,M.S., Hood,L., Hansen,J.A. and Petersdorf,E.W. (2002) Oligonucleotide arrays for high-throughput SNPs detection in the MHC class I genes: HLA-B as a model system. *Genome Res.*, **12**, 447–457.
5. Guo,Z., Guifoyle,R.A., Thiel,A., Wang,R. and Smith,L.M. (1994) Direct fluorescence analysis of genetic polymorhisms by hybridization with

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oligonucleotide arrays on glass supports. *Nucleic Acids Res.*, **22**, 5456–5465.
6. Zammatteo,N., Jeanmart,L., Hamels,S., Coutois,S., Louette,P., Hevesi,L. and Remacle,J. (2000) Comparison between different strategies of covalent attachment of DNA to glass surfaces to build DNA microarrays. *Anal. Biochem.*, **280**, 143–150.
7. Peterson,A.W., Heaton,R.J. and Georgiadis,R.M. (2001) The effect of surface probe density on DNA hybridization. *Nucleic Acids Res.*, **29**, 5163–5168.
8. Beier,M. and Hoheisel,J.D. (1999) Versatile derivatisation of solid support media for covalent bonding on DNA microchips. *Nucleic Acids Res.*, **27**, 1970–1977.
9. Shchepinov,M.S., Case-Green,S.C. and Southern,E. (1997) Steric factors influencing hybridisation of nucleic acids to oligonucleotide arrays. *Nucleic Acids Res.*, **25**, 1155–1161.
10. Wang,H.-Y., Malek,R.L., Kwitek,A.E., Greene,A.S., Luu,T.V., Behbahani,B., Frank,B., Quackenbush,J. and Lee,N.H. (2003) Assessing unmodified 70-mer oligonucleotide probe performance on glass-slide micorarrays. *Genome Biol.*, **4**, R5.
11. Metz,D.H. and Brown,G.L. (1969) The investigation of nucleic acid secondary structure by means of chemical modification with a carbodiimide reagent. I. The reaction between N-cyclohexyl-N′-β-(4-methylmorpholinium) ethylcarbodiimide and model nucleotide. *Biochemistry*, **8**, 2312–2328.
12. Kikuchi,J., Furukawa,Y., Suzuki,O., Hayashi,N., Nakamura,M., Morita,M. and Matsuda,M. (2000) A simple semisolid subtraction method using carbodiimide-coated microplates. *Mol. Biotechnol.*, **15**, 193–200.
13. Lee,J.-H., Hwang,G.-S., Kim,J.-K. and Choi,B.-S. (1998) The solution structure of DNA decamer duplex containing the Dewar product of thymidylyl(3′→5′)thymidine by NMR and full relaxation matrix refinement. *FEBS Lett.*, **428**, 269–274.
14. Jing,Y., Kao,J.F.-L. and Taylor,J.-S. (1998) Thermodynamic and base-pairing studies of matched and mismatched DNA dodecamer duplexes containing *cis-syn*, (6–4) and Dewar photoproducts of TT. *Nucleic Acids Res.*, **26**, 3845–3853.
15. Thonai,N., Inaki,Y., Miyata,M., Yasui,N., Mochizuki,E. and Kai,Y. (1998) Photodimerization of thymine derivatives in single crystal. *J. Photopolymer Sci. Technol.*, **11**, 59–64.
16. Taylor,J.-S., Brockie,I.R. and O'Day,C.L. (1987) A building block for the sequence-specific introduction of cis-syn thymine dimers into oligonucleotides. solid-phase synthesis of TpT[*c*,*s*]pTpT. *J. Am. Chem. Soc.*, **109**, 6735–6742.
17. Lingbeck,J.M. and Taylor,J.-S. (1999) Preparation and characterization of DNA containing a site-specific nonadjacent cyclobutane thymine dimer of the type implicated in UV-induced –1 frameshift mutagenesis. *Biochemistry*, **38**, 13717–13724.
18. Okubo,T., Harada,S., Higuchi,S. and Matsushima,S. (2002) Investigation of quantitative trait loci in the CCKAR gene with susceptibility to alcoholism. *Alcohol Clin. Exp. Res.*, **26** (Suppl.), 2S–5S.
19. Rawson,E.S., Nolan,A., Silver,K., Shuldiner,A.R. and Poehlman,E.T. (2002) No effect of the Trp64Arg beta(3)-adrenoceptor gene variant on weight loss, body composition, or energy expenditure in obese, Caucasian postmenopausal women. *Metabolism*, **51**, 801–806.
20. Exton,M.S., Artz,M., Siffert,W. and Schedlowski,M. (2003) G protein beta3 subunit 825T allele is associated with depression in young, healthy subjects. *Neuroreport*, **14**, 531–533.
21. Herrmann,S.M., Ringel,J., Wang,J.G., Staessen,J.A. and Brand,E. (2002) Peroxisome proliferator-activated receptor-gamma2 polymorphism Pro12Ala is associated with nephropathy in type 2 diabetes: The Berlin Diabetes Mellitus (BeDiaM) Study. *Diabetes*, **51**, 2653–2657.
22. Hamer,O.W., Forstner,D., Ottinger,I., Ristow,M., Bollheimer,L.C., Scholmerich,J. and Palitzsch,K.D. (2002) The Pro115Gln polymorphism within the PPAR gamma2 gene has no epidemiological impact on morbid obesity. *Exp. Clin. Endocrinol. Diabetes*., **110**, 230–234.
23. Cheung,V.G., Morley,M., Aguilar,F., Massimi,A., Kucherlapati,R. and Childs,G. (1999) Making and reading micorarrays. *Nature Genet.*, **21** (Suppl.), 15–19.
24. Wang,X. and Seed,B. (2003) Selection of oligonucleotide probes for protein coding sequences. *Bioinformatics*, **19**, 796–802.

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