Research Report

Fabrication of DNA Microarrays Using Unmodified Oligonucleotide Probes

ABSTRACT

Microarrays printed on glass slides are often constructed by covalently linking oligonucleotide probes to a derivatized surface. These procedures typically require relatively expensive amine- or thiol-modified oligonucleotide probes that add considerable expense to larger arrays. We describe a system by which unmodified oligonucleotide probes are bound to either non-derivatized or epoxy-silane-derivatized glass slides. Biotinylated PCR products are heat denatured, hybridized to the arrays, and detected using an enzymatic amplification system. Unmodified probes appear to detach from the slide surface at high pH (> 10.0), suggesting that hydrogen bonding plays a significant role in probe attachment. Regardless of surface preparation, high temperature (up to 65°C) and low ionic strength (deionized water) do not disturb probe attachment; hence, the fabrication method described here is suitable for a wide range of hybridization stringencies and conditions. We illustrate kinetics of room temperature hybridizations for probes attached to nonderivatized slides, and we demonstrate that unmodified probes produce hybridization signals equal to amine-modified, covalently bound probes. Our method provides a cost-effective alternative to conventional attachment strategies that is particularly suitable for genotyping PCR products with nucleic acid microarrays.

INTRODUCTION

Nucleic acid microarrays are frequently used for expression profiling (12,16), genotyping (7), DNA sequencing (14), diagnostics (6,18), immunology (9), and drug discovery and development (4). The general paradigm involves hybridizing a labeled nucleic acid “target” to a complementary “probe” (e.g., oligonucleotide, peptide nucleic acid, cDNA, PCR product) that has been attached to a glass, membrane, or gel pad substrate. Oligonucleotide probes can be synthesized on glass surfaces in situ (12), or they can be synthesized ex situ and covalently attached to the substrate (8,11). In the latter case, probes are covalently attached to a derivatized surface through a primary amine or thiol (1–3) or via homo- or heterofunctional linkers (2). In an alternative approach, Doktycz and Beattie (5) coupled 3′-propanolamine-derivatized oligonucleotides directly to unmodified glass surfaces. While effective, probe modifications add considerable expense to the microarray, especially for the individual researcher printing in relatively low volumes for multiple projects. Likewise, surface derivatization with silanes involves the use, hazards, and expense of several toxic compounds.

When our laboratory began constructing microarrays with amine-modified probes, we found that it was unnecessary to derivatize glass slides for probe attachment and that probe modification was immaterial for binding to the slide surface (under the conditions that we employ). We hypothesized that successful array manufacture and target hybridization was a consequence of stable hydrogen bonding or electrostatic attraction between the probe and the glass surface. In this paper, we present evidence that is consistent with this hypothesis and demonstrate the utility of unmodified probes for microarray hybridizations. We compare the performance of unmodified and amine-modified oligonucleotide microarrays and define optimal hybridization and detection conditions for the unmodified probes. In so doing, we demonstrate that arrays constructed from unmodified probes are robust and suitable for microarray protocols and offer significant cost savings for microarray development and production.

MATERIALS AND METHODS

Materials

Twelve-well, Teflon®-masked slides were obtained from Eri Scientific (Portsmouth, NH, USA). 3-Glycidoxypropyltrimethoxysilane (epoxy-silane; Sigma-Aldrich, Milwaukee, WI, USA) was used as received. All oligonucleotides were purchased from Midland Certified Reagent (Midland, TX, USA). Amine-modified oligonucleotides were purified by reversed-phase HPLC, and unmodified or biotinylated oligonucleotides were purified by gel filtration. Peptide nucleic acids (PNAs) were purchased from Applied Biosystems (Foster City, CA, USA). Enzyme-labeled fluorescence (ELF®-97) was used for target detection. The ELF-97 substrate and wash buffers were adopted from an in situ mRNA hybridization kit (Molecular Probes, Eugene, OR, USA). AMDEX streptavidin-alkaline phosphatase (SA-AP) conjugate was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).
Surface Preparation and Spotting

Glass slides were washed in mild detergent followed by successive 10-min acid baths (3 M H$_2$SO$_4$ and 3 M HCl) and rinsed in deionized water. Slides used for noncovalent bonding were dried under a nitrogen stream and used for array printing without further modification. For epoxy-silane derivatization (“epoxy-silanized”), acid-washed slides were dried as above and then incubated in a 50-mL polypropylene tube with 2% epoxy-silane in methanol (HPLC grade). After 15 min, slides were removed and double washed in 100% methanol, followed by immediate drying under a nitrogen stream. Both slide treatments appeared effective when stored for several days at room temperature. Oligonucleotide probes were resuspended (typically 100 µM) in printing buffer (0.01% SDS, 50 mM NaOH, pH 12.0) and spotted in triplicate onto the prepared slides using a Model 417 Arrayer (Genetic Microsystems, Woburn, MA, USA). The spotting protocol included two 20-s washes (water) with 10-s vacuum drying after each wash step. Each spot was approximately 150 µm in size. After printing, slides were baked for 30–60 min at 130°C in a vacuum oven and stored at 4°C.

Probes and Targets

A 36-mer quality-control (QC) probe and complementary target were adopted from Lamture et al. (11). The QC probe was manufactured both with and without a 3’ amine-modification (5’-TTGT-GGTGGTGTTGGTGGGTTGGGTGGTG-3’) and the complementary QC target incorporated a 3’ biotin. A capture probe for the eaeA locus of E. coli O157:H7 (5’-TCAAGAGTTGCCCATCCTGCAGCAA-3’) (13) was synthesized with and without a 3’ amine-modification. We also used a biotinylated version of the eaeA probe, and for one experiment we used a 5’ biotinylated PNA (5’-OOOOGGCCACATCGACTCCCGT-3’). Genomic DNA was isolated from E. coli O157:H7 (ATCC strain no. 51657) by phenol: chloroform extraction (15). PCR products (104 bp) for the eaeA locus were generated from genomic DNA using 5’ biotinylated primers (5’-AAAGTTCA GATCTTGATGACATTG-3’ and 5’-CAATTTCAGGGAATAACATTG-3’), and HotStarTaq™ (Qiagen, Valencia, CA, USA) with three-cycle PCR [denaturation at 95°C for 15 s, annealing at 58°C for 45 s, and extension at 72°C for 45 s; 35 cycles; Model 9600 thermal cycler (Applied Biosystems)]. Final reaction conditions were 1× PCR buffer (Qiagen), 4 mM MgCl$_2$, 200 nM each dNTP, 2 U HotStarTaq, 50–300 ng genomic DNA. PCR products (104 bp) were purified and biotinylated. Oligonucleotide probes were resuspended (typically 100 µM) in printing buffer (0.01% SDS, 50 mM NaOH, pH 12.0) and spotted in triplicate onto the prepared slides using a Model 417 Arrayer (Genetic Microsystems, Woburn, MA, USA). The spotting protocol included two 20-s washes (water) with 10-s vacuum drying after each wash step. Each spot was approximately 150 µm in size. After printing, slides were baked for 30–60 min at 130°C in a vacuum oven and stored at 4°C.
DNA in a total volume of 50 µL. In most cases, PCR products were hybridized directly onto the arrays without further purification. We also concentrated PCR products by pooling reactions \((n = 20)\), precipitating (15) and quantifying by spectrophotometry.

**Hybridization and Detection**

All hybridization steps for this study were carried out at either room temperature or 37°C in a humidified chamber. The general protocol included blocking nonspecific binding sites using hybridization buffer (5× Denhardt’s solution, 150 mM sodium citrate) for 30 min (35 µL/well). Targets (typically 4 µL PCR products and 0.57 µM QC target in hybridization buffer) were heat denatured for 5 min in hybridization buffer and then chilled to 4°C. Blocking buffer was removed from the slide wells by aspiration, and chilled targets were hybridized onto the arrays for 60–120 min at 23°C or 37°C. After hybridization, targets were removed by aspiration, and slides were washed in 1×SSC (150 mM NaCl, 0.15 mM sodium citrate, pH 7.0). Residual fluid was aspirated from the slide surface, and SA-AP (1:500 in hybridization buffer) was incubated on the slides for 30 min. Slides were then washed in 1× ELF-97 wash buffer, and 20 µL ELF-97 substrate were incubated in each well for 30–60 min (1:100 in ELF-97 developing buffer). Slides were given a final wash (50 mM NaCl, 0.1% Tween® 20, 100 mM Tris, pH 8.0), followed by two rinses in deionized water, and air-dried. Slides were illuminated with UV (290–365 nm), and fluorescent emissions (520 nm) were quantified as optical density (OD) using a Fluor-S MultiImager (Bio-Rad Laboratories, Hercules, CA, USA). The imager was equipped with a 28–200 mm DL Hyperzoom Macro lens (Sigma, Rödermark, Germany) that was fitted with a +1 close-up lens. Images were quantified with Phoretix array software version 1.00 (Phoretix International, Newcastle, UK). Hybridization signals (OD) were presented as raw values that were calculated as the mean of three triplicate spots within a single well. For statistical purposes, individual wells were considered replicates, and statistical calculations were made with NCSS 2000 (10).

**Probe Stability**

To evaluate the stability of probe attachment, we first subjected arrays to a pH gradient. Amine-modified QC probes were printed on epoxy-silanized or acid-washed slides and then pre-soaked for 2 h in 1× SSC at pH 1.0 to pH 13.0. QC targets were then hybridized to the arrays as described above. To test the effects of ionic strength on probe attachment, amine-modified (QC) and unmodified \((eaeA)\) probes were printed on epoxy-silanized and acid-washed slides followed by 2-h, room temperature incubations in either deionized water or 4 M NaCl (pH 7.0). Appropriate targets were then hybridized to the arrays as described above. Temperature effects were evaluated by printing both amine-modified and unmodified \(eaeA\) probes on epoxy-silanized and acid-washed slides. These slides were then pre-soaked two hours with 1× SSC (pH 7.0) at 23°C or 65°C followed by hybridization of targets and detection. Stability of probes over time was evaluated by printing unmodified \(eaeA\) and amine-modified \(eaeA\) on acid-washed and epoxy-silanized slides, respectively. Slides were then pre-soaked for 0, 1, 2, 3, 4, and 16 h at 23°C in 1× SSC (pH 7.0) followed by target hybridization and detection.

**Hybridization Kinetics**

To characterize the kinetics of PCR product and QC target hybridizations to unmodified probes on acid-washed

![Figure 1](image1.png)

**Figure 1.** Effect of epoxy-silane concentration on QC hybridization signal. Derivatized slides were prepared using tenfold dilutions (450 mM to 4.5 nM) of epoxy-silane. Amine-modified QC probes were printed on slides, and QC targets were hybridized using room temperature conditions. There was no significant change in hybridization signal with increasing concentration of derivatizing epoxy-silane solution \((r = -0.43; P = 0.1)\).

![Figure 2](image2.png)

**Figure 2.** Room temperature hybridization experiment with unmodified probes printed on nonderivatized, acid-washed slides. Each well has a lower row of QC probes and an upper row of \(eaeA\) probes. (A) Hybridization included both \(eaeA\) PCR products and QC target. (B) Hybridization included \(eaeA\) PCR products only. (C) Hybridization included positive control targets only. Biotinylated QC probe carryover (if present) would be detected as a second row of faint spots in panel C.
slides, we conducted a series of room temperature hybridization experiments using a range of incubation times for denaturation, hybridization, SA-AP, and ELF-97 development. Only one variable was changed per experiment, and results were gauged by the intensity of the fluorescent signal after ELF-97 development. Only one variable was changed per experiment, and results were gauged by the intensity of the fluorescent signal after ELF-97 development. PCR mixture was applied to individual wells with a single PCR (50 μL plus buffer) divided between 12 individual wells (one slide). Products were denatured for 0, 1, 2, 4, 8, or 10 min before hybridization. Subsequent experiments used 5-min heat denaturation followed by hybridization for 5, 10, 20, 30, 60, and 120 min (with 30 min SA-AP and 60 min ELF), or SA-AP incubations for 2, 5, 10, 15, 20, and 30 min (with 2 h hybridizations, 60 min ELF-97), or ELF-97 incubations for 5, 10, 20, 30, 45, or 60 min (with 2 h hybridization, 30 min SA-AP).

**Probe Sensitivity**

To examine how probe attachment might affect detection sensitivity at different target concentrations, we hybridized eaeA PCR products at “low” (3 nM) and “high” (94 nM) concentrations to unmodified probes on acid-washed slides or to amine-modified probes on epoxy-silanized slides. We used a “short” protocol (30 min hybridization, 15 min SA-AP, 30 min ELF-97) that was intended for the relatively rapid detection of PCR products with some loss of sensitivity and a “long” protocol (120 min hybridization, 30 min SA-AP, 60 min ELF-97) to enhance detection sensitivity. Experiments were conducted at 23°C and at 37°C.

**RESULTS**

**Probe Attachment**

To determine the optimum concentration of epoxy-silane needed for covalent attachment of amine-modified probes, we derivatized a series of slides with tenfold dilutions of epoxy-silane. After hybridization, we found no significant change in signal intensity with increasing concentration of epoxy-silane (r = -0.43, P = 0.1) (Figure 1).

Oligonucleotide probe modifications (amine, biotin, or no modification) had no effect on the ability to bind probes to nonderivatized, Teflon-masked slides. Unmodified probes could also be printed and hybridized on standard, non-derivatized, unmasked slides (Fisher Scientific, Pittsburgh, PA, USA) (data not shown), indicating that epoxysilane treatment was not necessary for stable probe attachment and the Teflon masking process had no obvious effect on glass surface chemistry. Consequently, we printed unmodified oligonucleotide probes (100 μM) on acid-washed slides and were able to easily detect QC targets (36-mer) and eaeA PCR products (104 bp) without cross-hybridization between targets and probes (Figure 2) and no detectable probe carryover during the printing process (Figure 2C).

To evaluate the effect of probe concentration on signal intensity, we printed a range of eaeA probe concentrations on unmodified and silanized slide surfaces. Strong hybridization signals were evident for all probe concentrations with peak signals between 60 and 100 μM (Figure 3). The greatest signal intensity was achieved when printing unmodified probes on epoxy-silane-derivatized slides. For this study, we did not pursue the question of enhanced signal intensity on silanized surfaces further and instead printed probes at 100 μM for all subsequent experiments (with acid-washed and silanized surfaces).

**Probe Stability**

QC hybridization signal for amine-modified probes attached to silanized surfaces was unaffected by extreme pHs, whereas hybridization signal for probes that were printed on acid-washed slides began declining rapidly when arrays were pre-soaked with alkaline buffer (pH > 10.0) (Figure 4). Probe stability (as measured by QC or eaeA hybridization signal intensity) was also unaffected by pre-soaking arrays in deionized water versus 4 M NaCl, a result that was obtained for both silanized (P = 0.25) and acid-washed surfaces (P = 0.17).

To test the effects of hybridization temperature on probe stability, printed arrays were pre-soaked at 23°C or 65°C.

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**Figure 3.** Effect of oligonucleotide probe concentration on hybridization signal for eaeA PCR products. Unmodified probes (closed circle) or amino-modified probes (open circle) were printed on epoxy-silane-derivatized slides (solid line) or acid-washed slides (dashed line). PCR products were hybridized to the arrays for 2 h (37°C). Data represent average from five independent arrays per treatment combination.
for 2 h. Hybridization signal for both probe types (amine-modified versus unmodified) were statistically identical for each slide type (acid-washed and silanized) and temperature treatment ($P = 0.50$) (Figure 5). Again, probes printed on epoxy-silanized slides produced significantly greater hybridization signals compared with probes printed on acid-washed slides ($P < 0.0001$; compare with Figure 3). Presoaking acid-washed and silanized slides at 65°C also significantly enhanced hybridization signal compared with slides that were presoaked at 23°C ($P < 0.0001$).

When we examined the time-dependent stability of probe attachment, we found no significant differences in hybridization signal attributable to soaking time (0–16 h) for either unmodified or amine-modified probes printed on acid-washed or silanized surfaces ($P > 0.18$, $r = 0.07$, and $r = 0.17$, respectively).

We have successfully hybridized other PCR targets to unmodified, 15-mer probes printed on acid-washed slides (data not shown). The $eaeA$ PCR product has also been hybridized to a 10-mer probe but only when this probe was amine-modified and printed on an epoxy-silane surface (data not shown).

To test for possible effects of electrostatic charge on probe attachment, we also printed biotinylated PNA (print buffer, pH 9.0) and oligonucleotide probes on both acid-washed and epoxy-silanized slides. These slides were then treated with SA-AP (1 h) followed by standard ELF-97 development. The biotinylated PNA was clearly present on both slide surfaces, and the signal intensity was comparable with the signal obtained from the biotinylated oligonucleotide (data not shown).

Hybridization Kinetics

While it is possible to detect native PCR products, heat denaturing (95 degrees) for as little as 1 min was sufficient to double hybridization signal ($P = 0.02$). Longer denaturation times yielded no significant improvement in hybridization signal ($r = 0.05$, $P = 0.8$). Allowing more time for targets to anneal to probes improved signal intensity continuously through 120 min (Figure 6A). For the SA-AP and ELF-97 reactions, QC approached the maximum detection limit of our scanning device (OD = 155 648), whereas hybridization signal from the PCR products did not approach saturation in the allotted time (Figure 6, B and C).

Sensitivity

Single-factor analysis of variance (ANOVA) showed no difference in hybridization signal intensity between....

![Figure 4. Effect of pH on functionality of amine-modified QC probes on epoxy-silanized slides (open circles; covalent attachment) and on acid-washed slides (Closed circles; noncovalent attachment). Each point represents average signal intensity from two independent experiments.](image)

![Figure 5. Effect of temperature on eaeA probe functionality. Acid-washed slides (solid bar) and epoxy-silanized slides (open bar) were printed with amine-modified and unmodified eaeA probes. Slides were presoaked for 2 h in 1× SSC (pH 7.0) at 23°C or 65°C followed by hybridization and detection of eaeA PCR products. Bars represent average (± SEM) for five replicate arrays per treatment combination.)](image)
probe types (unmodified probe on glass versus amine-modified probe on epoxysilane) for either low or high target concentrations ($P = 0.54$ and $P = 0.95$, respectively). The dynamics of hybridization differed between protocol lengths and hybridization temperatures. Regardless of target concentration, either a longer hybridization protocol or $37^\circ C$ treatment produced significantly greater signal intensity relative to the shorter protocol and room temperature hybridizations ($P < 0.016$ and $P < 0.0001$, respectively) (Figure 7). The temperature effect was quite dramatic for low target concentrations where signal intensity increased over eightfold, whereas it only increased 3.3-fold for higher target concentrations. For both target concentrations, warming the reaction with the short protocol produced greater signal intensity than the long protocol at room temperature (Figure 7). These results demonstrate a simple means to enhance detection sensitivity while simultaneously reducing the time needed for detecting PCR products.

**DISCUSSION**

While nucleic acid adsorption to nonderivatized glass is not a novel phenomenon, we demonstrate that unmodified oligonucleotide probes are stably bound to unmodified glass slides and are capable of binding their complementary sequences within the context of a microarray system. Our method offers considerable cost savings over conventional attachment chemistry, particularly if the latter requires purification after oligonucleotide modification. Our results also suggest that probes can be printed at concentrations as low as 20 nM, although the actual probe concentration used will depend on the degree of sensitivity required and the target concentration.

The actual mechanism by which unmodified probes adhere to the slide surface (acid-washed or silanized) was not identified in this study. Nevertheless, we hypothesize that attachment involves noncovalent interactions with the respective surfaces, either as electrostatic attraction between the negatively charged phosphate backbone of the oligonucleotide and positively charged...
surface ions or the result of hydrogen bonding between the oligonucleotide and Si-OH groups (acid-washed slides) or hydrolyzed epoxy groups (epoxy-silanized slides). Two lines of evidence suggest a more prominent role for hydrogen bonding. First, excessively alkaline conditions (pH > 11.5) deprotonate nucleotides and thus break hydrogen bonds (19). Results in Figure 4 indicate that amine-modified probes detach from acid-washed slide surfaces at higher pH (> 10.0), while the same probes remain bound to epoxy-silanized slides where they are expected to be covalently attached to the surface and thus unaffected by the range of pH used in this experiment. Second, if electrostatic charge is the primary mechanism for probe attachment, then the neutrally charged backbone of a PNA (17) would be less likely to bind to acid-washed glass, although the amine terminus of the PNA should bind covalently to the epoxy-silanized surface. To test this, we printed biotinylated PNA on both acid-washed and epoxy-silane slides and found that the PNA adhered well to both surfaces. While these tests are not definitive, they suggest that hydrogen bonding is a primary mechanism for attachment of unmodified probes to acid-washed or silanized glass surfaces. If hydrogen bonding and electrostatic attraction are the primary mechanisms of probe attachment, then we can expect probe attachment to be less robust for shorter oligonucleotides, although we have successfully used probes as short as 15-mer for identification of single-nucleotide polymorphisms.

While we focused our investigation on simple, acid-washed slides, hybridization signal was significantly enhanced for both unmodified and amine-modified probes printed on epoxy-silane surfaces (Figure 3 and Figure 5). This result may be due to the more flexible nature of the epoxy-silane molecule relative to probe attachment via bonding to the Si-OH surface. Presoaking acid-washed or silanized arrays at 65°C further enhanced hybridization signal intensity (Figure 5). Presoaking the slide surface may positively influence probe stability, but these conditions also appear to reduce the hydrophilic nature of both slide surfaces. Consequently, we hypothesize that the presoaking treatment enhances the 3-D target diffusion to the glass surface and subsequent 2-D interaction with immobilized probes (20).

Whether or not the observed differences in signal intensity between acid-washed and epoxy-silanized surfaces are practically significant depends on the particular application of the microarray and experimental protocol. That is, the increase in hybridization signal intensity on silanized slides was less than two times the signal obtained on acid-washed slides (Figure 3 and Figure 5). With a typical microarray experiment consisting of high concentrations of PCR product, a greater than two times increase in detection sensitivity is probably immaterial, and practitioners can take advantage of addi-
tional cost savings (both reagent and waste disposal) by avoiding the epoxy-silane treatment. Applications requiring exquisite detection sensitivity may be better served with other microarray printing procedures.

Regardless of the mechanism of probe attachment or differences in signal intensity between acid-washed and silanized glass surfaces, our basic microarray printing and hybridization protocol is sufficient to detect and discriminate between distinct nucleic acid targets. The experiments described here were performed at room temperature or 37°C, but we have used this system to simultaneously distinguish between four distinct PCR products using both room temperature and 50°C hybridizations (unpublished data). Likewise, the acid-washed, unmodified probe attachment strategy is compatible with a range of ionic concentrations and temperatures (0–4 M NaCl; ambient temperature to 65°C). We have also successfully hybridized a 633-bp eaeA PCR product to unmodified probes on acid-washed slides, indicating that this printing process and strategy is not limited to capturing short PCR products or oligonucleotide targets. For the longer probes used in this study (≥25-mer), the cost savings afforded by using unmodified probes did not require any sacrifice in hybridization signal strength when compared with an equivalent, amine-modified probe. Preliminary experiments also indicate that Cy3-labeled eaeA products will hybridize to unmodified probes and detection sensitivity is comparable with the ELF-97 system described here. Thus, the printing method described here should also be compatible with fluorescent labeling/detection strategies in common usage and provide a significant cost savings for manufacturing microarrays on glass surfaces.

REFERENCES


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