Title: IMPROVED SUPPORT FOR SOLID PHASE HYBRIDIZATION ASSAYS

Hybridization signal from oligonucleotide probes. 5'-OH PCR probes, and 5'-Acryl-ylc™ PCR probes
X-axis: supports, with probe concentrations [pM] as spotting solution
Y-axis: hybridization signal per spot, Relative Fluorescence Units

Control
Boiling for 2 min
Boiling for 10 min
Boiling for 60 min

(57) Abstract: A method of immobilizing a nucleic acid on a solid support and the resulting support containing the immobilized nucleic acid. The solid support has at least one immobilized thiol group, which reacts with and binds a nucleic acid to immobilized the nucleic acid on the support. The thiol group can be rendered unreactive, and then can be reactivated to bind the nucleic acid. The method is applicable for use with either double stranded or single stranded nucleic acid, and can be used to bind oligonucleotides and/or polymerase chain reaction products.
IMPROVED SUPPORT FOR SOLID PHASE HYBRIDIZATION ASSAYS

This application claims the benefit of United States Patent Application Serial No. 60/236,287, filed on September 28, 2000.

FIELD OF THE INVENTION

The invention relates generally to a nucleic acid microarray composition and method, and more specifically to a microarray containing thiol reactive groups.

BACKGROUND OF THE INVENTION

DNA microarray technology is becoming an increasingly important research tool in biomedical sciences. For instance, microarray technology is a very popular method for analyzing the expression of thousands of genes in parallel within a single sample. Probes for many mRNA species (or their cDNA counterparts) are spotted or synthesized in situ at discrete locations on a planar substrate. The array is then contacted with a labeled RNA or cDNA sample. Probe spots that hybridize are detected and quantified to analyze the level of expression of each gene for which a probe (or set of probes) was arrayed.
For many researchers, the usual substrate is a glass microscope slide that has been treated with coatings to improve the binding of the probes to the slide surface. Since nucleic acids have large negative charge density, coatings with a positive charge such as poly-lysine or aminopropylsilane are popular. After spotting, the slides are baked and/or irradiated with ultraviolet light to crosslink the probes to the slide coating.

Synthetic oligonucleotides and polymerase chain reaction (PCR) products are commonly used as probes. Each probe type has strengths and weaknesses. PCR products are popular for analysis of gene expression, because they may be generated for all clones in a given library using a single primer set that hybridizes to common vector sequences flanking the inserted cDNA sequence. Moreover, cDNA libraries are easy to construct, and many previously characterized cDNA libraries and individual cDNA clones are available in the research community and from commercial sources.

Despite these advantages, the use of PCR products as probes is complicated by several factors. PCR products bind readily to aminopropylsilane and poly-lysine coated slides. However, many investigators find considerable variation in the efficiency and stability of PCR product probe binding to different slides, and even between different regions of a single slide.

Often, this variability requires running replicate microarray experiments numerous times to ensure that the correct hybridization signal is measured. This is inconvenient, and preparation of large numbers of PCR products is expensive and laborious.
Oligonucleotide probes are easy to make in large amounts and to characterize. However, attachment to poly-lysine and aminopropylsilane coated supports is very inefficient, and most workers using such probes utilize specific linking chemistries. For example, TeleChem International, Inc. (Sunnyvale, CA) and SurModics, Inc. (Eden Prairie, MN) sell slides coated with specific chemical groups (aldehydes and N-hydroxysuccinimide esters, respectively) that will react specifically with the primary amines on amine-modified oligonucleotides. One problem with these supports is that the probe loading density is not as high as with the positively charged supports. However, the major problem with the use of oligonucleotide probes is that hybridization performance is more unpredictable than PCR probe performance.

From the previous discussion, it is apparent that reproducible, reliable methods and improved substrates for fabricating microarrays are needed. It would be especially advantageous to the field to have a single support type that could be used with oligonucleotides or PCR products. In addition, it would be useful to be able to bind single or double-stranded probes with similar efficiency. Finally, it would be advantageous to have improved supports that would permit attachment of unmodified probe nucleic acids, in the absence of any specialized chemical moieties such as, for example, 5'-amine or biotin groups.

**SUMMARY OF THE INVENTION**

The present invention is directed to improved substrates and methods for attaching nucleic acids to solid supports. In particular, the invention is especially useful for fabrication of nucleic acid hybridization arrays,
also known in the literature as microarrays or DNA microarrays. The invention
is based on the discovery that DNA molecules bind stably to surfaces coated
with a high concentration of thiol groups.

In one aspect the invention is directed to a method of immobilizing

5 a nucleic acid on a solid support. A solid support contains an immobilized thiol
group, which is contacted with a nucleic acid and forms a bond between the
thiol group and the nucleic acid, thereby immobilizing the nucleic acid on the
solid support. The nucleic acid can be an unmodified nucleic acid, a modified
nucleic acid or a nucleic acid analog. The nucleic acid can be single-stranded

10 or double-stranded. The solid support can include a plurality of thiol groups
and a plurality of nucleic acids immobilized on the solid support. The solid
support can be formed from a compound including a glass, plastic, ceramic or
metal. The solid support can include two or more spatially distinct regions,
each region including a plurality of immobilized nucleic acids. Each spatially

15 distinct region can contain a different type of nucleic acid. The solid support
can be a microarray. Alternatively, the solid support can be treated with thiol-
reactive reagents to convert any unreacted thiol group to an unreactive form.

In another embodiment, the invention is directed to providing a

20 solid support including an immobilized latent thiol group, activating the latent
thiol group, and reacting the activated thiol group with a nucleic acid, thereby
immobilizing a nucleic acid on a solid support. The steps of activating the latent
thiol groups and reacting the activated thiol groups can occur essentially
simultaneously. The nucleic acids can be an unmodified nucleic acid, a
modified nucleic acid or a nucleic acid analog. The nucleic acids can be single-
stranded or double-stranded. The solid support can include a plurality of thiol
groups and a plurality of nucleic acids immobilized on the solid support. The
solid support can be formed from a compound including a glass, plastic,
ceramic or metal. The solid support can include two or more spatially distinct
regions, each region including a plurality of immobilized nucleic acids. Each
spatially distinct region can contain a different type of nucleic acid. The solid
support can be a microarray. In another aspect, the invention is directed to the
product formed by the method of the invention.

In another embodiment, the invention is directed to a method of
producing a microarray comprising a plurality of single-stranded nucleic acids
including providing a solid support comprising a plurality of immobilized thiol
groups, contacting the thiol groups with double-stranded nucleic acids, forming
a bond between the thiol groups and the double-stranded nucleic acids such
that the double-stranded nucleic acids are immobilized on the microarray, and
denaturing the double-stranded immobilized nucleic acids, such that
immobilized single-stranded nucleic acids are formed, thereby, producing a
microarray comprising a plurality of single-stranded nucleic acids. Alternatively,
the double-stranded nucleic acids may be denatured before deposition on the
solid support, converting the double-stranded nucleic acids to single-stranded
forms prior to immobilization.

In another aspect, the invention is directed to a kit for attaching
nucleic acids to a solid support comprising a solid support component
comprising a plurality of immobilized latent thiol groups and instructions for
activating the thiol groups to form bonds with nucleic acids. The kit can also
include at least one additional component including an activator component, a
blocking component, and a wash buffer component.

In yet another aspect, the invention is directed to a method for
detecting or separating target nucleic acids from other components contained in
a sample. A microarray has a plurality of immobilized thiol groups, which are
contacted with a plurality of nucleic acids that contain sequences
complementary to a subsequence of the nucleotide sequence of the target
nucleic acid. A bond forms between the thiol group and the nucleic acids,
thereby immobilizing the nucleic acids on the solid support. The immobilized
nucleic acids are contacted with the sample, and target nucleic acids hybridize
with immobilized nucleic acids with complementary subsequences, thereby
detecting or separating target nucleic acids from other components contained in
the sample.

The invention is also directed to a nucleic acid microarray. A solid
support contains immobilized thiol groups that are reacted with and bind to
nucleic acids to immobilize the nucleic acids. The microarray can contain
oligonucleotides and/or products of the polymerization chain reaction.

The foregoing and other objects, features and advantages of the
invention will be apparent from the following more particular description of
preferred embodiments of the invention, as illustrated in the accompanying
drawings, detailed description, and examples.

**BRIEF DESCRIPTION OF THE DRAWINGS**

FIG.1a is an image of the hybridization signals from an array in
which probes were not denatured *in situ* prior to hybridization.
FIG. 1b is an image of the hybridization signals from an array in which probes were denatured *in situ* by boiling for 2 minutes prior to hybridization.

FIG. 1c is an image of the hybridization signals from an array in which probes were denatured *in situ* by boiling for 10 minutes prior to hybridization.

FIG. 1d is an image of the hybridization signals from an array in which probes were denatured *in situ* by boiling for 60 minutes prior to hybridization.

FIG. 2 is a bar graph comparing hybridization signals from oligonucleotide probes and PCR probes in varying concentrations in solution.

FIG. 3 is a bar graph comparing hybridization signals from oligonucleotide probes with various concentrations in solution.

FIG. 4 is a bar graph comparing hybridization signals from oligonucleotide probes with various concentrations in solution.

**DETAILED DESCRIPTION OF THE INVENTION**

In one embodiment of the invention, DNA probes are deposited onto a solid support that have surface thiol groups. Optionally, unreacted thiol groups can be capped with excess thiol reactive compound. The slides are washed and dried for storage until use.

Preferred probes for use in the inventive method include double-stranded and single-stranded nucleic acids.

The term "modified nucleic acid" is intended to include nucleic acids containing modified bases, deoxyribose/ribose groups or phosphates, or
modified linkages, e.g., 2'-5' deoxyribo or ribonucleic acids. Examples of nucleic acids having modified bases include, for example, acetylated, carboxylated, or methylated bases (e.g., 4-acetylcytidine, 5-carboxymethylaminomethyluridine, 1-methylinosine).

The term “unmodified nucleic acid” is intended to include nucleic acids that do not contain any specialized chemical moieties, such as modified bases, deoxyribose/ribose groups, or phosphates.

The term “nucleic acid analog” is intended to include molecules that lack a conventional deoxyribose/ribose-phosphodiester backbone, but which retain the ability to form Watson-Crick type base pairs with complementary single-stranded nucleic acids. Examples of nucleic acid analogues include peptide nucleic acids (PNAS; Egholm et al., J. Am. Chem. Soc. (1992) 114:1895-1897) and morpholino oligomers (morpholinos; Summerton and Weller, Antisense Nucleic acid Drug Dev., (1997) 7:187-195).

It will be apparent to those skilled in the art that similar design strategies can be used to construct other nucleic acid analogs that will have useful properties for immobilized probe assays.

Useful single-stranded probes include synthetic oligonucleotides, denatured PCR products, denatured recombinant plasmids, or recombinant single-stranded bacterial, viral, or phage nucleic acids. Double-stranded probes include PCR products, recombinant plasmids, recombinant phage genomes, or restriction fragments produced from recombinant plasmids or phage genomes. Useful PCR products may be amplified from genomic DNA or recombinant clones. Such recombinant DNA clones may include those
produced from genomic DNA, total RNA, mRNA, or cDNA. Many cDNA libraries and cDNA clones are available commercially and from the literature, and PCR products produced from these clones are particularly useful as probes for the analysis of gene expression.

When using double-stranded probes, the double-stranded probes may be subjected to denaturing treatments before deposition on the support to convert the double-stranded probes to single-stranded forms. Alternatively, the double-stranded probes may be denatured in situ after deposition and binding on the support. For example, probe-derivatized supports can be heated in low ionic strength aqueous solutions or water at 90-100°C for approximately 10 seconds to 60 minutes. As is well known in the literature, other denaturation conditions can be used. For example, by including chemical denaturants in the denaturation solution, lower wash temperatures can be used to achieve denaturation.

Suitable solid supports include glass, plastic, ceramic, and metal materials that have surface thiol groups. Planar shapes, such as microscope slides, are particularly useful for microarray applications. For other applications, other shapes may also be useful. For example, supports in the form of beads, microspheres, meshes, membranes, porous filters, woven matrices, tubes, cups, multiwell plates, track-etch membranes, posts, fibers, optical fibers, are all suitable for the methods of the invention.

Thiol groups can be provided by coating the supports with thiol containing materials such as thiol silanes, thiol-containing monomers and polymers, or disulfide-containing silanes and polymers, such as
poly(mercaptopropyl)methyl siloxane, 3-mercaptopropyl trimethoxysilane,
mercaptomethylmethyl diethoxysilane, 3-mercaptopropylmethyl
dimethoxysilane, N-mercapto ethylacrylamide, N,N, csytamine bisacrylamide,
and 4-[[1-Oxo-3-[[2-[(1-oxo-2-propenyl)-
high stability of binding is important, it is useful if the thiol-containing coating is
covalently bonded to the support. For example, a mercaptosilane derivative
may be covalently bound to glass via standard silanization methods known in
the art. In addition, thiol or disulfide acryl monomer or polymer derivatives may
be coupled to glass via an acryl-modified silane derivative using free-radical
polymerizations. Polymerization methods known in the art may be used to
couple thiol or disulfide acryl monomeric derivatives to certain plastic supports,
for example PMMA, for covalent attachment.

Supports include glass and plastic supports that are coated with
polymeric materials that have thiol groups. Especially preferred supports have
chemically protected thiol groups that can be deprotected or activated after
storage and prior to probe deposition. One preferred protected thiol group is a
disulfide group, which can be activated by treatment with reducing agents such
as dithiothreitol (DTT), beta-mercaptoethanol (BME), and tris-carboxy ethyl
phosphine (TCEP). Several preferred coatings, including disulfide coatings, are
described in United States application Serial Nos. 60/151,267 (filed 8/27/99),
60/177,844 (filed 1/25/00) and 09/649,837 (filed 8/28/00), the entire teachings
of which are hereby incorporated by reference in their entireties.
Polymeric materials for coating the supports include, but are not limited to, acrylamide and derivatized acrylamides. Acrylamide-based coatings incorporating disulfide acrylamides, such as bisacryloylacrylamide and AEMA, are especially useful for the invention. Use of such polymeric coatings are described in United States applications Serial Nos. 60/151,267 (filed 8/27/99), 60/177,844 (filed 1/25/00) and 09/649,637 (filed 8/28/00).

In one embodiment of the invention, the probes are single-stranded oligonucleotides. In another embodiment of the invention, the probes are spotted as double stranded DNA molecules and converted to a single stranded form after spotting and binding to the support. In one preferred embodiment of the invention, the probes are double-stranded PCR products, which are spotted in double-stranded form and denatured after binding to the support.

One embodiment of the invention is an improved method for fabricating microarrays using PCR products as probes comprising the following steps:

1. Provide a planar support with a surface layer comprising thiol groups.

2. Contact the support with one or more PCR products which will serve as hybridization probes to allow binding of the PCR product to the support.

3. Optionally, treat the support with thiol-reactive reagents to convert any unreacted thiol group to an unreactive form.
4. Denature PCR products by heating in low ionic strength buffers at 90-100°C for 2-60 minutes.

5. Wash and dry the denatured supports.

In one preferred embodiment of the invention, the substrate is a glass slide which is coated with a polymer layer that comprises disulfide groups. The polymer layer can be treated with a reducing agent which converts disulfide groups to thiol groups prior to or simultaneously with PCR product spotting.

The ability to use one support for attachment of both oligonucleotides and PCR probes is an important improvement for the microarray field.

The methods of the invention are exemplified by the following non-limiting examples:

**EXAMPLE 1**

Preparation of glass supports with disulfide-containing polymeric coating (BAC-coated slides)

Materials and vendors for reagents:

1) BAC (N,N'-bis(acryloyl)cystamine) - Sigma Chemical, St. Louis, MO, A-4929 (5 g)

2) TCEP (tris-(carboxyethyl)phosphine hydrochloride) - Sigma Chemical #C-4706

3) DMF (N,N'-dimethyl formamide) - American Bioanalytical, Natick, MA, #AB00450
4) APS (ammonium persulfate) and TEMED (N,N,N',N'-tetramethylethlenediamine) - Bio-Rad, Richmond, CA, #161-0700 and #161-0800 respectively.

5) Poly(ethylene glycol) 400 monomethyl ether monomethacrylate - Polysciences, Warrington, PA #16665.

6) Acrylic Acid - Sigma-Aldrich, St. Louis, MO #14,723-0.

7) TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA buffer) - Sigma Chemical, #T-9285.

8) SSPE (saline sodium phosphate-EDTA) - Fisher Scientific, #BP1328-1.

9) TWEEN (Polyoxyethylene-sorbitan monolaurate) - Sigma Chemical, #P-1379.

10) N-Lauroyl Sarkosine - Sigma Chemical, St. Louis, MO, L5777.

11) Methacryloxypropyl trimethoxy silane - Gelest, Tullytown, PA, #SIM6487-4.

12) Sodium Hydroxide - Fisher Scientific, Pittsburgh, PA, #SS255.


14) Alcohol, denatured - American Bioanalytical, Natick, MA, #AB00137.

15) Sodium Bicarbonate - Sigma Chemical, St. Louis, MO, #56297.

Procedure for slide fabrication and activation:

1) Glass microscope slides were cleaned and coated with an acrylic silane layer using methods known in the art. (ACR-25C). Fifty slides were placed in a custom vertical polypropylene slide holder (Andy's Machine, Middleboro, MA).

2) The following solution was prepared and filtered using a Whatman #3 paper filter (Aldrich, Milwaukee, WI #Z24,022-2):

- 103 ml DMF
- 3.5 g BAC
- 3.5 ml PEG 400 monomethylethermonomethacrylate
- 57.0 ml Millipore water

3) 5.4 ml of 10% APS (pre-filtered using Millex-GP, 0.22 μm, SLGPR 25KS) and 100 μl of TEMED was added to the solution of step 2.

   Final concentrations are 2% BAC, 2% PEG 400 monomethylethermonomethacrylate, and 60% DMF.

   The solution was mixed and poured into a slide holder containing the acrylic silanized slides. Polymerization of the coating layer was allowed to proceed for up to 12 hours at room temperature.

4) After polymerization the cloudy solution phase was
decanted and the slides were rinsed with deionized water and dried.

5) Prior to probe spotting, slides were activated by soaking them in a slide container containing 50 mM TCEP (TCEP was freshly prepared in deionized water and filtered using a Whatman Millex-GP filter prior to use). Slides were soaked for 15 min with frequent agitation.

6) Reduced slides were washed briefly in two changes of deionized water and dried under compressed filtered air. Activated dried slides could be used stored for one month under ambient conditions without apparent loss of binding activity.

EXAMPLE 2

Fabrication of microarrays using PCR product probes on BAC-coated slides

15 Probes

PCR product probes:

Rabbit reticulocyte first strand cDNA was prepared from rabbit reticulocyte polyA(+) mRNA (Gibco-BRL; Life Technologies; Rockville, MD) with an array TRACKER™ Standard Labeling cDNA Kit (Display Systems Biotech, Inc., Vista, CA 490-100) using the kit manufacturer's instructions. An aliquot of the cDNA preparation was used in a PCR reaction using the primers:

1. Forward primer, 5'-GTCCAGTGAGGAGAAGTCT-3' SEQ ID NO: 1, and

2. Reverse primer, 5'-CCAGAAGTCAGATGCTCA-3' SEQ ID NO: 2,
which amplify a 498 bp region from the 3' end of the beta-globin mRNA sequence, as shown below.

Sequence of the rabbit beta-globin PCR product (primer sites shown in bold).

5  GTCCAGTGAGGAGAAGTCTGCAGTCACTGCCCCCTGTTGGGCAAGGTGAAT
    GTGGAAGAAAGCTTGTTGGTGAAGGCCCGGTGGCAGGC
    TGCTGGTGTCTACCCATGGACCCAGAGGTTTCTCGAGTCCTTTGGGAC
    CTGTCCTCTGCAATGCTGGTATGA
    ACAATCCTAAGGTGAAGGGCTCATGGCAAGAAGGTTGCTCGTGCCTCAGT

10  GAGGGTCTGAGTCACCTGGACAAC
    CTCAAAAGGCACCTTTGCTAAGCTGTAAGCTGAACGTGACACTGTGACAAGCTGCA
    CGTGATCTGAGAACATTCAGGCT
    CCTGGGCAACGTGCTGGTTATTTGTCGTCTCATTTCATTTGGCACAAGATT
    CACTCCTCAGGTGCAGGCTGCCTA

15  TCAGAAGGTGTTGCTGGCTGGCGCAATGCGCCTGGTCACAAATACCACT
    GAGATCTTTTTCTCCTGCAAAAATTATGGGACATCATGAAGCCCCT
    TGAGCATCTGACTTTCTGG

SEQ ID NO: 3.

Two batches of rabbit beta-globin PCR products were prepared.

20  One batch used ordinary 5'-hydroxyl primers. The second batch used a forward primer with a 5'-acrylamide modification. The 5'-acrylamide group was added during primer synthesis using an acrylamide phosphoramidite (Acrydite™ phosphoramidite, Matrix Technologies, Waltham, MA). The 5'-acrylamide group readily forms a thioether bond with thiol groups, as described in U.S.
applications Serial Nos. 60/177,844 (filed 1/25/00) and 09/649,637 (filed 8/28/00).

To provide a negative control PCR product probe, a ~300 base pair PCR product from the human ApoE gene was amplified from human genomic DNA and was processed in parallel with the rabbit beta-globin PCR products. The ApoE gene was amplified using an Acrydite™-modified forward primer.

PCR products were purified using the QIAquick PCR purification kit (Qiagen, Chatsworth, CA). Following purification, the samples were ethanol precipitated, and the quality and concentration of the PCR products was estimated by polyacrylamide gel electrophoresis. Concentrations were estimated by densitometric comparisons of the fluorescent staining intensity of the PCR products and plasmid restriction fragment size standards of known concentration.

Acrydite™-modified oligonucleotide probes:

For purposes of comparison, Acrydite™-modified oligonucleotide probes, which bind very efficiently to reduced BAC-coated slides, were spotted on the same slides used for the PCR product probes. The sequence of the rabbit beta-globin probe is (BG1216-70; 70mer probe):

5′-Acrydite™-TGA GAT CTT TTT CCC TCT GCC AAA AAT TAT GGG GAC ATC ATG AAG CCC CTT GAG CAT CTG ACT TCT GGC T-3′  SEQ ID NO: 4

A negative control oligonucleotide probe, which was not expected to hybridize with rabbit beta-globin cDNA or mouse cardiac cDNA, was also spotted on the BAC-coated slides along with the rabbit beta-globin probes.
(Acrydite™-modified oligonucleotide complementary to 16 S bacterial rRNA):
5'-Acrydite™-GAA TTC CAG GTG TAG CGG TGA AAT GCG TAG AGA TCT
GGA GGA ATA CCG GTG GCG AAG GCG GCC CCC TGG AC-3'
SEQ ID NO: 5

5 Spotting of BAC-coated slides:

A series of solutions of 40 µl volume was prepared for spotting on slides. All solutions were in 100 mM sodium carbonate pH 10 buffer containing 0.05% of sodium dodecyl sulfate (SDS). The solutions were placed in microplate wells (Microseal 96 V-bottom microplates, MJ Research, MA) and the arrays were spotted using GMS 417 Arrayer, according to manufacturer’s suggestions (Affymetrix, Santa Clara, CA).

After spotting, the slides were treated to modify any unreacted thiol groups by soaking in 1 M sodium acrylate (sodium salt of acrylic acid) in 100 mM sodium carbonate buffer pH 10) for 20 min. After quenching, the slides were washed twice with 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 200 mM NaCl, and twice with 10 mM Tris-HCl pH 8.0, 1 mM EDTA (TE buffer), and dried.

To denature the PCR products, spotted quenched slides were treated in boiling deionized water for 0 (control, unboiled), 2, 10, or 60 minutes, and dried.

Hybridization and Analysis

Cy3-dUTP labeled cDNA was prepared from rabbit reticulocyte polyA(+) mRNA (Gibco-BRL; Life Technologies; Rockville, MD) and from mouse cardiac polyA(+) RNA (Clontech, Palo Alto, CA) with an array
TRACKER™ Standard Labeling cDNA Kit (Display Systems Biotech, Inc., Vista, CA 490-100). The kit manufacturer's instructions were followed, except that after the final precipitation, the cDNA preparation was resuspended in 40 µL of buffer (4X SSPE; 0.02% Tween20), and this mixture was run through a G25 spin column (Amersham Pharmacia, Microspin G-25 column, 27-5325-01).

Hybridization was carried out overnight at 55°C in plastic hybridization chambers (HybriWell, Grace Bio-Labs Inc., Bend, OR) with 10 nanograms of Cy3-labeled rabbit globin cDNA and 100 nanograms of Cy3-labeled mouse cardiac cDNA per 100 ml of hybridization buffer (4x SSPE containing 0.02% Tween 20). After hybridization the slides were washed three times in 1x SSPE buffer and two times in TE buffer. Washed slides were dried under a stream of nitrogen gas.

The slides were scanned with a ScanArray 4000 scanner (GSI Lumonics, Billerica, MA) using the green line (543.5 nm) of the HeNe laser for excitation. The laser power was set at 90% and photomultiplier power (PMT) was set at 60%. The data were analyzed using ImageQuant 5.1 software (Molecular Dynamics, Sunnyvale, CA).

Images of the arrays are shown in FIGS. 1a-1d. The integrated intensity for each spot of the array as determined using ImageQuant 5.1 software (Molecular Dynamics, Sunnyvale, CA). Strong hybridization of the beta-globin cDNA was observed to the beta-globin oligonucleotide probes in all experiments. Hybridization to the beta-globin PCR products was greatly enhanced by the boiling treatment. The hybridization was highly specific in all cases as shown by the fact that negative control probes (16S rRNA
oligonucleotide probes and the human ApoE PCR probes) showed no
detectable hybridization signals. The stability of probe binding is shown by the
fact that strong hybridization signals were obtained from slides that had been
denatured by boiling for as long as 1 hour.

The averaged data for the total signal per spot for selected array
spots are shown in FIG. 2. The oligonucleotide concentrations used for
spotting ranged form 10 to 1000 nanomolar for the Acrydite™-modified beta-
globin probe, but the PCR probes covered a smaller range from approximately
10 to 240 nanomolar.

Analysis of FIGS. 1a, 1b, and 2 shows that the Acrydite™-modified
oligonucleotide globin probes show relatively high signals with or without array
denaturation. However, both PCR globin probes show good performance after
denaturation. The PCR probes show 2-5 higher signals than the
oligonucleotide probes at similar concentrations.

Acrydite™ modification appears to have only a small effect on
efficiency of PCR probe binding, as assessed by the cDNA hybridization
signals. Similar hybridization signals are seen from both Acrydite™-modified
and unmodified PCR probes. This is in contrast to the affect of Acrydite™
modification on some oligonucleotide probes, where Acrydite™-modified
oligonucleotides can give significantly better hybridization signals than
unmodified probe oligonucleotides of the same base sequence (see below and
United States applications Serial Nos. 60/151,267 (filed 8/27/99), 60/177,844
(filed 1/25/00), and 09/649,637 (filed 8/28/00).
Acrydite™-modified PCR probes appear to have slightly better thermal stability than unmodified PCR probes as seen in the data from the arrays that were subjected to 10 and 60 minute denaturations (FIGS. 2 and 3).

**EXAMPLE 3**

5 Use of oligonucleotide probes to fabricate microarrays on thiol-coated surfaces

Two different oligonucleotide probes were synthesized in 5'-Acrydite™-modified form (designated as ac in the FIGS.), 5'-primary amine form (designated as NH₂ in the FIGS.), and unmodified form (5'-hydroxyl form):

- ANF401-70 (mouse atrial natriuretic factor probe 70mer)
- 5'-ATC TGC CCT CTT GAA AAG CAA ACT GAG GGC TCT GCT CGC TGG CCC TCG GAG CCT ACG AAG ATC CAG CTG C-3’
  SEQ ID NO: 6
- BG1236-50 (rabbit β-globin probe 50mer)
- 5'-CCA AAA ATT ATG GGG ACA TCA TGA AGC CCC TTG AGC ATC TGA CTT CTG GCT-3’
  SEQ ID NO: 7

Oligonucleotides were dissolved at 10 and 30 micromolar concentrations in 100 mM carbonate buffer pH 10, 0.01% sarkosyl, and spotted onto BAC-coated slides as described in Example 2.

Slides were hybridized with a mixture of 5.0 ng of Cy3-labeled rabbit reticulocyte cDNA spiked into 1.0 μg of Cy3-labeled mouse cardiac cDNA and analyzed as described in Example 2.

The results are shown in FIGS. 3 and 4. In both cases, Acrydite™-modified probes give higher hybridization signals than amine-modified or
unmodified oligos. However, amine-modified and unmodified probes still give substantial hybridization signals, suggesting that at least two modes of probe binding to the support are occurring, only one of which is Acrydite™-dependent.

Taken together with the PCR probe data of FIG. 2, these data demonstrate that an Acrydite™-independent probe binding process is operative when unmodified nucleic acid probes are deposited on supports having surface thiol groups. For short oligonucleotide probes, this process appears to be less efficient than the Acrydite™-dependent process. However, with PCR probes, which are longer, the Acrydite™-independent process appears to be at least as efficient as the Acrydite™-dependent process.

FIGS. 3 and 4 also demonstrate that oligonucleotide probes modified with primary amine groups show slight improvements in hybridization performance relative to unmodified probes. This demonstrates that the primary amine group may participate in a binding reaction with the thiol surface that is distinguishable from that of the Acrydite™-modified and unmodified probes.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.
What is claimed is:

1. A method of immobilizing a nucleic acid on a solid support comprising providing a solid support having an immobilized thiol group thereon, and contacting the solid support with a nucleic acid to react and form a bond between the thiol group and the nucleic acid to immobilize the nucleic acid on the solid support.

2. The method of claim 1 wherein the nucleic acid is selected from the group consisting of an unmodified nucleic acid, a modified nucleic acid, and a nucleic acid analog.

3. The method of claim 1 wherein the nucleic acid is an oligonucleotide.

4. The method of claim 1 wherein the nucleic acid is a polymerase chain reaction product.

5. The method of claim 1 wherein the nucleic acid is double-stranded.

6. The method of claim 1, wherein the nucleic acid is single-stranded.
7. The method of claim 1 further comprising contacting the solid support with a reagent containing a latent thiol to provide a solid support having an immobilized latent thiol group thereon, activating the latent thiol group, and contacting the solid support with a nucleic acid to react and form a bond between the thiol group and the nucleic acid to immobilize the nucleic acid on the solid support.

8. The method of claim 1 wherein the immobilized nucleic acid forms a microarray.
9. A method of immobilizing a nucleic acid on a solid support comprising
   providing a solid support having an immobilized latent thiol
   group thereon, activating the latent thiol group, and contacting the solid support
   with a nucleic acid to react and form a bond between the thiol group and the
   nucleic acid to immobilize the nucleic acid on the solid support.

10. The method of claim 9 wherein the nucleic acid is selected from
    the group consisting of an unmodified nucleic acid, a modified nucleic acid, and
    a nucleic acid analog.

11. The method of claim 9 wherein the thiol group is activated and
    reacted with the nucleic acid upon contact of the solid support with the nucleic
    acid.

12. The method of claim 9 wherein the nucleic acid is selected from
    the group consisting of an oligonucleotide, a polymerase chain reaction
    product, and combinations thereof.

13. The method of claim 9 wherein the nucleic acid is double-
    stranded.

14. The method of claim 9 wherein the nucleic acid is single-stranded.
15. The method of claim 9 wherein the latent thiol groups are thiol-containing materials selected from the group consisting of thiol silanes, thiol-containing monomers and polymers, disulfide-containing silanes and polymers, and combinations thereof.

16. The method of claim 12 wherein the immobilized nucleic acid forms a microarray.
17. A method of producing a microarray having a plurality of single-stranded nucleic acids comprising

providing a solid support having a plurality of immobilized thiol groups thereon;

5 contacting the solid support with a plurality of double-stranded nucleic acids;

immobilizing the double-stranded nucleic acids on the microarray by reacting the thiol groups with the double-stranded nucleic acids to form a bond therebetween; and

10 denaturing the double-stranded immobilized nucleic acids to form single-stranded nucleic acids to produce a microarray having a plurality of single-stranded nucleic acids.

18. A method of claim 17 wherein the double-stranded nucleic acids are denatured prior to contact with the solid support, such that a plurality of single-stranded nucleic acids react to form a bond with the immobilized thiol groups.

19. The method of claim 17 wherein the double-stranded nucleic acids are selected from the group consisting of oligonucleotides, polymerase chain reaction products, and combinations thereof.
20. A kit for immobilizing a plurality of nucleic acids on a solid support comprising a solid support having a plurality of immobilized latent thiol groups thereon, and instructions for activating the thiol groups to form bonds with the nucleic acids.

21. The kit of claim 20 further comprising at least one component selected from the group consisting of an activator component, a latent thiol-containing component, and a wash buffer.

22. The kit of claim 21 wherein the activator component is selected from the group consisting of dithiothreitol, beta-mercaptoethanol, tris-carboxyethyl phosphine, and combinations thereof.

23. The kit of claim 21 wherein the latent thiol-containing component is selected from the group consisting of N,N'-bis(acryloyl)cystamine and 4-[[1-Oxo-3-[[2-[(1-oxo-2-propenyl)-amino]ethyl]dithio]propyl]amino]butanoic acid.
24. A method for isolating a target nucleic acid in a sample comprising

providing a solid support having at least one nucleic acid immobilized via reacting and forming a bond with at least one immobilized thiol group, said nucleic acid having a nucleotide sequence complementary to a target nucleic acid, and

contacting the immobilized nucleic acid with the sample under conditions sufficient to allow hybridization of the target nucleic acid to the immobilized nucleic acid to isolate the target nucleic acid from the sample.

25. The method of claim 24 wherein the solid support comprises a microarray.
26. A nucleic acid microarray comprising a solid support having a plurality of immobilized thiol group thereon reacted and bonded with a plurality of nucleic acids to immobilize the nucleic acids to the solid support.

27. The microarray of claim 26 wherein the thiol groups are thiol-containing materials selected from the group consisting of thiol silanes, thiol-containing monomers and polymers, disulfide-containing silanes and polymers, and combinations thereof.

28. The microarray of claim 26 wherein the thiol groups are latent and are thereafter activated to be capable of reacting and bonding with the nucleic acid.

29. The microarray of claim 26 wherein the solid support is formed from a compound selected from the group consisting of glass, plastic, ceramic, and metal.

30. The microarray of claim 26 wherein the solid support comprises two or more spatially distinct regions, each region immobilizing at least one nucleic acid.
31. The microarray of claim 26 wherein spatially distinct regions on the solid support immobilize a nucleic acid selected from the group consisting of an oligonucleotide, a polymerase chain reaction product, and combinations thereof.

32. The microarray of claim 26 wherein the nucleic acid is selected from the group consisting of a double stranded nucleic acid, a single stranded nucleic acid, and combinations thereof.
Spotted probes:
BG = rabbit beta-globin oligo probe
16S = 16S rRNA oligo probe
Reg = globin PCR probe - regular primers
Ac = globin PCR probe - Acrydite™ primers
(+) = complementary beta-globin cDNA
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Panel of probes spotted in triplicate sets

Conc. of probe in spotting soln. [nM]:

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Figure 1a.
Spotted probes:
BG = rabbit beta-globin oligo probe
16S = 16S rRNA oligo probe
Reg = globin PCR probe - regular primers
Ac = globin PCR probe - Acrydite™ primers
(+) = complementary beta-globin cDNA

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Panel of probes spotted in triplicate sets

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Figure 1b.
Spotted probes:
BG = rabbit beta-globin oligo probe
16S = 16S rRNA oligo probe
Reg = globin PCR probe - regular primers
Ac = globin PCR probe - Acrydite™ primers
(+)= complementary beta-globin cDNA

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Panel of probes spotted in triplicate sets

Conc. of probe in spotting soln. [nM]:

| 1000 | 10⁴ | 10⁴ | -- | 3.5 | 17 |
| 100  | 1   | 2   | 9  | 44  | 220 |
| 10   | --  | 2   | 10 | 52  | 260 |

Probes denatured in situ by boiling for 10 min. prior to hybridization.

Figure 1c.
Spotted probes:
BG = rabbit beta-globin oligo probe
16S = 16S rRNA oligo probe
Reg = globin PCR probe - régular primers
Ac = globin PCR probe - Acrydite™ primers
(+) = complementary beta-globin cDNA

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Panel of probes spotted in triplicate sets

Conc. of probe in spotting soln. [nM]:

| 1000 | 10^4 | 10^4 | -- | 3.5 | 17  |
| 100  | 1    | 2    | 9  | 44  | 220 |
| 10   | --   | 2    | 10 | 52  | 260 |

Probes denatured in situ by boiling for 60 min. prior to hybridization.

Figure 1d.
Hybridization signal from oligonucleotide probes, 5'-OH PCR probes, and 5'-Acrydite™ PCR probes

X-axis numbers indicate probe concentrations [nM] in spotting solution

Figure 2.
Oligonucleotide probe & Spotting conc.

Figure 3.
Figure 4.

Oligonucleotide Probe & Spotting Conc.

Hybridization Signal (Relative Fluorescence units)
<110> Patterson, Brian C.
Mielewczyk, Slawomir
Maurer, Anthony J.

<120> Improved Support for Solid Phase Hybridization Assays

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