Technical Parameters for the Use of Corning® DNA-BIND™ Products in High-Throughput Screening

Application Note

Innovative Techniques in Drug Discovery

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Summary

Corning offers a line of products for the covalent immobilization of biomolecules to enable the development of a wide variety of high-throughput solid-phase assays. DNA-BIND 96-well plates, 1 x 8 Stripwell™ plates and Thermowell® M PCR plates have surfaces with covalently-linked N-oxysuccinimide esters (referred to as NOS groups) that react with nucleophiles such as primary amines. Kinetic analysis demonstrates that immobilization of amine C-12 modified oligonucleotides occurs predominantly in the first 10 minutes of the reaction at room temperature and that more rapid coupling occurs with higher concentrations of aminated oligonucleotides. Further evaluation of the parameters for the use of these products indicates that the reactivity of the NOS groups to primary amine containing molecules is undiminished 24 hours after exposure to room air. Subjecting the immobilized oligonucleotide to common reagents used for cell lysis or denaturation also has little effect on the signal obtained following hybridization. Using the DNA-BIND Thermowell M plate for solid-phase PCR, a ratio of 1:12.5 liquid-phase to solid-phase forward primer provides the greatest amount of PCR product in both the solid and liquid-phase.

Introduction

Since the onset of recombinant DNA technology in the 1970s, the specificity of homologous strands of DNA has allowed for the detection of nucleic acids with specifically labeled probes (1). The need for simpler, more rapid analysis techniques has driven the development of solid-phase assays for analysis of a multitude of protein, DNA and RNA targets (2-4). While membrane hybridizations require much longer incubation and washing times to allow reagents to permeate into the membrane, the use of microplates in solid-phase assays are generally much more rapid and easier to handle. However, microplates used in many solid-phase assays require the use of carbodiimide coupling reagents or UV irradiation for covalent immobilization (2-4). Unlike other microplates used in solid-phase assays, Corning DNA-BIND 96-well plates, 1 x 8 Stripwell plates and Thermowell M PCR plates require no activation. These products have surfaces with covalently-linked N-oxysuccinimide (NOS) esters that quickly react with primary amine groups at slightly alkaline pH. The NOS ester undergoes nucleophilic substitution as the amine attacks the carbonyl group and displaces the N-oxysuccinimide group (Figure 1). The resulting amide linkage covalently couples primary amine containing molecules to the surface of DNA-BIND 96-well plates, 1 x 8 Stripwell plates and Thermowell M PCR plates.

While these plates were specifically designed for the immobilization of aminated DNA for use in nucleic acid hybridization assays and solid-phase PCR, peptides and other small primary amine containing molecules can also be coupled to the surface. This amine reactive chemistry enables the development of a wide variety of assays for high-throughput screening. Previous work has demonstrated the use of DNA-BIND products for hybridization to immobilized capture probes (5), solid-phase mini-sequencing (6), examining DNA-protein interactions (7), and kinase assays (8). This surface is not recommended for binding large protein mole-

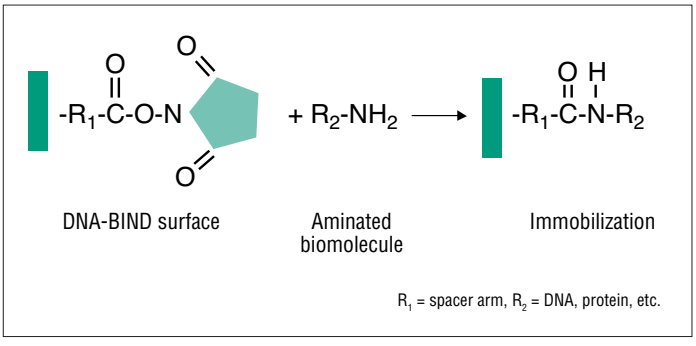

Figure 1. Reaction of N-oxysuccinimide with an aminated biomolecule.

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cules since they usually contain many amines that react with the DNA-BIND™ surface. This may result in insufficient biological activity of the immobilized molecule due to conformational changes, inaccessibility of functional sites, or steric hindrance.

DNA with primary amines, added synthetically or by *in vitro* manipulation, can be directly coupled to the NOS surface. One of the most common mechanisms for modifying DNA with a primary amine group is to incorporate the amine onto the 5' or 3' end of the molecule via a 12 carbon linker during synthesis. The linker extends the oligonucleotide away from the surface of the plate to reduce steric hindrance and provide greater access to homologous nucleic acids in solution. Electron resonance in the ring structure of the DNA bases stabilizes these amino groups, and makes them less reactive with the NOS surface, although conditions can be altered to encourage this interaction.

Materials and Methods

Oligonucleotide Preparation

Oligonucleotides were purchased from BioSource International (Foster City, CA). A doubly modified oligonucleotide with an amine 12 carbon linker at the 5' end and fluorescein at the 3' end (Amine C12 5' GATATGCAGAGCATATAAAATGAGGTAGGA 3'-fluorescein) was used to determine coupling kinetics and NOS surface reactivity over time. Complementary sequences (Amine C12 5' GATATGCAGAGCATATAAAATGAGGTAGGA 3' capture oligo and fluorescein-5' ATATCTCCTACCTCATTTTATATGCTCTGC 3' hybridizing oligo) were used for hybridization experiments including stability of immobilized oligonucleotides following exposure to cellular denaturants. Oligonucleotide sequences derived from the Beta actin gene (accession #x00351 J00074 M10278) were used as primers for solid-phase PCR (Amine C12 5' ATCCGCCGCCCGTCCACA 3' = forward primer (capture oligo) and fluorescein- 5' AGCAATGCTATCACCTCCCCTGTG 3' =reverse primer). Capture oligonucleotides were diluted with coupling buffer [500 mM Na_2HPO_4, pH 8.5, (Sigma Chemical, Cat. No. S-9763) 1 mM ethylenediamine tetraacetic acid (EDTA, Fisher Scientific, Cat. No. BP119-500)] for immobilization. The hybridization oligonucleotide was diluted with hybridization buffer [5x Standard Saline Citrate (SSC), diluted from a 20x SSC stock which is 0.3 M Na Citrate, pH 7, 3.0 M NaCl (Gibco BRL, Cat. No. 15557044), and 0.1% sodium dodecyl sulfate (SDS, Sigma, Cat. No. 39,714-8)] prior to use.

Kinetic Analysis of Immobilization

Doubly modified oligonucleotide prepared as stated above, was diluted to 1, 10, and 100 pmole per 100 µL of coupling buffer and allowed to incubate for 0, 5, 10, 30, and 60 minutes in wells of DNA-BIND plates (Corning, Cat. No. 2505). Wells with coupling buffer only served as controls. Following incubation at room temperature (RT) for the allotted time, the wells were rinsed three times with 10 mM TRIS, pH 8.0, 150 mM NaCl (TBS, Sigma, Cat. No. T-8524). Fluorescence was detected in relative fluorescence units (RFUs) using the fluorescence intensity method in an LJL Biosystems Analyst™ (Sunnyvale, CA) with the following settings: digital conversion, attenuator, out; z height, 2.0 mm; excitation filter, 485 nm; emission filter, 530 nm. The wells were aspirated to remove the TBS, and TBS, pH 8, with 0.05% Tween 20 (Fisher, Cat. No. BP337-100), and 1% hammerstein casein (EM Scientific, Cat. No. CX0525-3) (block buffer) was added to all wells. Following a 15 minute incubation at RT, the block buffer was removed and sheep anti-fluorescein, horseradish peroxidase-conjugated antibody (Roche Molecular Biochemicals, Cat. No. 1426346) diluted 1:2000 in block buffer was added to all wells and incubated for 30 minutes at RT. Wells were rinsed three times with TBS, pH 8, 0.05% Tween 20 (TTBS). Tetramethylbenzadine (TMB) substrate (Sigma, Cat. No. T-3405) in 0.05 M phosphate-citrate buffer, pH 5 was added. Following a 15 minute incubation at RT, the enzymatic reaction was terminated with the addition of 25 µL of 2 M sulfuric acid (Sigma, Cat. No. S1526). Absorbance was measured at 450 nm on a SpectraMax™ 250 (Molecular Devices Corp., Sunnyvale, CA).

Detection of Hybridization at Increasing Oligonucleotide Concentrations

Capture oligonucleotide at concentrations of 0, 0.5, 5, 50, 100 and 250 pmole per 100 µL of coupling buffer was incubated in wells of DNA-BIND plates (Corning, Cat. No. 2505) for 15 minutes at RT. Wells were rinsed three times with TBS and incubated for 15 minutes at RT with block buffer. Fluoresceinated hybridizing oligonucleotide, in 5x SSC, pH 7.5, 0.1% SDS at concentrations equivalent to added capture oligonucleotide, was added to wells and incubated for 30 minutes at 55°C. Following hybridization, wells were rinsed three times with post-hybridization wash (0.1xSSC/ 0.1% SDS) and incubated with block buffer for 15 minutes at RT. Block buffer was removed and hybridization was detected using a sheep anti-fluorescein, horseradish peroxidase conjugated antibody as previously described. Following incubation with enzyme substrate, absorbance was measured at 450 nm on a SpectraMax 250.

Direct and Indirect Detection-Coupled Oligonucleotides

Doubly modified oligonucleotide at concentrations of 0, 5, 50, and 100 pmole per 100 µL of coupling buffer was incubated in wells of DNA-BIND solid black microplates (Corning, Cat. No. 2498) for 15 minutes at RT. Wells were rinsed three times with TBS and fluorescence was measured on an LJL Biosystems Analyst™ (Sunnyvale, CA.) at the previously stated settings. Wash buffer was aspirated, wells were blocked, and fluorescence was detected using a sheep anti-fluorescein, horseradish peroxidase-conjugated antibody as previously described. Following incubation with the enzyme substrate, the solutions were transferred to a clear, general assay plate (Corning, Cat. No. 3628) and absorbance was measured at 450 nm on a SpectraMax 250.

Reactivity of the DNA-BIND™ Surface

To test the reactivity of the DNA-BIND NOS groups with primary amine containing molecules over time, DNA-Bind black opaque microplates (Corning, Cat. No. 2498) were removed from their foil wrappers, covered with universal lids (Corning, Cat. No. 3099), and incubated at RT for 0, 3, 6, and 24 hours. The doubly modified oligonucleotide was diluted to 50 pmole per 100 µL of coupling buffer and bound to wells of each plate. Following incubations, wells were washed, blocked, and fluorescence detected using an LJL Biosystems Analyst as previously stated.

Stability After Exposure to Common Denaturants

Capture oligonucleotide was diluted to 50 pmole per 100 µL of coupling buffer and incubated 15 minutes at RT in wells of DNA-BIND plates (Corning, Cat. No. 2505). Wells were rinsed three times with TBS and the following reagents were added to wells containing immobilized oligonucleotide: 6M guanidine (Fisher, Cat. No. BP-2211) in ddH_2O, 6M urea (Fisher, Cat. No. BP169500) in ddH_2O, 10% Triton X-100 (Sigma, Cat. No. T-9284) in ddH_2O, 10% SDS in ddH_2O, 50% dimethylformamide (Fisher, Cat. No. D119-500) in ddH_2O, Proteinase K (140 µg/mL, Roche Molecular Biochemicals, Cat. No. 1373196) in TBS, pH 8, 10% Tween 20 in ddH_2O, and TBS, pH 8. Following a 1 hour incubation at RT, all wells were rinsed with TBS, pH 8 and blocked for 15 minutes at RT, with block buffer. Block buffer was removed and 50 pmole of fluoresceinated hybridizing oligonucleotide per 100 µL of 5x SSC, pH 7.5, 0.1% SDS was added to all wells and incubated for 30 minutes at 55°C. Following hybridization, wells were rinsed with post-hybridization wash and incubated with block buffer for 15 minutes at RT. Block buffer was removed and hybridization was detected using a sheep anti-fluorescein, horseradish peroxidase-conjugated antibody as previously described. Following incubation with the enzyme substrate, absorb-ance was measured at 450 nm on a SpectraMax 250.

Solid-Phase PCR

Capture oligonucleotide was diluted to 50 pmole per 100 µL of coupling buffer and incubated 15 minutes at RT in wells of DNA-BIND Thermowell® M PCR plates (Corning Cat. No. 6573). Wells were rinsed three times with TBS and incubated 15 minutes at RT in block buffer. The block buffer was removed, and all wells were washed with TBS. Polymerase chain reactions (PCR) were prepared to compare the ratio of the coupled capture oligonucleotide (forward primer) to the same oligonucleotide in liquid phase. For each reaction, a master mix was made using the components of the GeneAmp™ PCR Kit (PE Biosystems, Foster City, CA, Cat. No. N801-0055). Final concentrations of components included were: 1x PCR buffer, 200 µM of each dNTP, 50 ng/100 µL of Beta actin Control Probe (Clontech, Palo Alto, CA, Cat. No. 9800-1), 25 mU/µL of AmpliTaq® DNA Polymerase, 50 pmol/100 µL of reverse primer, and sterile deionized water to the appropriate volume. Also included was sufficient forward primer to create ratios of 1:5, 1:7.5, 1:10, 1:12.5, and 1:15 with the 50 pmol/100 µL added in the initial preparation of the DNA-BIND Thermowell M PCR plate for solid-phase PCR. The PCR mix was added to the prepared DNA-BIND Thermowell M PCR plate. PCR was run in a PTC-200 DNA Engine (MJ Research, Inc., Watertown, MA) set at the following parameters: 94°C for 5 minutes, 30 cycles at 94°C for 30 seconds + 55°C for 30 seconds + 72°C for 1 minute, with a final extension cycle at 72°C for 5 minutes. The PCR reaction containing the liquid phase PCR product was aspirated from the wells, mixed with sample buffer, and loaded on an Ultra Pure DNA Grade 1% Agarose (Bio-Rad, Cat. No. 162-0133) gel for 30 minutes. The gel was stained for 10 minutes with SYBR® Green 1 Nucleic Acid Stain (Molecular Probes, Cat. No. S-7563). Bands were detected on a FluorImager® 595 (Molecular Dynamics, Sunnyvale, CA). Wells of the DNA-BIND Thermowell M PCR plate were washed three times with post-hybridization wash then incubated with sheep anti-fluorescein, horseradish peroxidase-conjugated antibody as previously described. Following incubation with the enzyme substrate, absorbance was measured at 450 nm on a SpectraMax 250.

Results and Discussion

The actual coupling of an aminated molecule to the surface of DNA-BIND 96-well plates, 1x8 Stripwell™ plates and Thermowell M PCR plates is accomplished by mixing the molecule in a slightly alkaline, phosphate or carbonate buffer, pH 8.5 to 9.5. Buffers containing primary amines, such as TRIS, are to be avoided during this initial immobilization step. Unlike other chemistries, NOS is an activated ester and can be used for coupling without the addition of carbodiimide coupling reagents, such as EDC. The initial coupling event with the DNA-BIND™ surface occurs very rapidly. Kinetic analysis demonstrates that covalent linkage occurs predominantly within the first 10 minutes of the reaction at room temperature for all concentrations of nucleotide examined (Figure 2).

The rate at which the amine C-12 modified oligonucleotides couple to the DNA-BIND surface can also be influenced by changing the concentration of the oligonucleotide during the coupling reaction. At low nucleotide concentrations (1 pmole/well), the rate of coupling can be substantially lower than at concentrations of 100 pmoles/well (Figure 2). A greater concentration of aminated oligonucleotide can be used to attain rapid coupling of the oligonucleotide to the DNA-BIND surface. However, this will provide no enhancement of the ultimate signal obtained since there are a limited number of NOS groups available for coupling. Figure 3 demonstrates that the level of signal obtained following hybridization appears to plateau with greater than 50 pmoles in 100 µL of coupling buffer added to the DNA-BIND wells. The lower limit of hybridization detected will depend upon the specific activity of the labeled oligonucleotide and the type of detection system employed.

While detection of fluorophore-labeled DNA can occur

directly in opaque DNA-BIND™ plates, the proximity of the fluorescent molecules to one another can lead to quenching of the signal depending on the amount of label incorporated into each target DNA molecule, and the total number of target DNA molecules captured. Figure 4 demonstrates the results of experiments comparing direct and indirect methods of detection. Direct detection of increasing concentrations of target DNA molecules conjugated to a single fluorescein molecule decreased when greater than 50 pmoles of capture oligonucleotide was added to the DNA-BIND well, compared to the typical saturation curve seen with indirect detection using an enzyme conjugated anti-fluorescein antibody.

When aqueous aminated oligonucleotides are added to a DNA-BIND well, numerous nucleophilic reactions with the NOS groups can occur. The displacement of the primary amine for the NOS group is the predominant reaction, but water and nucleotide base amines will also react with NOS at a much slower rate. It is therefore recommended that the DNA-BIND products be used for coupling as soon as possible after opening, although minimal loss of reactivity is observed up to 24 hours after opening with no change in non-specific (background) binding if the plates are covered with a lid and left at room temperature (Figure 5).

Once the amino C-12 modified oligonucleotide is coupled to the DNA-BIND surface, it is stable after exposure to a variety of conditions. Following coupling, DNA-BIND Thermowell® M PCR plates undergo repeated exposure to elevated temperatures without loss of hybridization signal, indicating that the DNA-BIND surface is thermally stable. Oligonucleotides coupled to the DNA-BIND surface were still available for hybridization following exposure to common reagents used for cell lysis or denaturation, suggesting that the DNA-BIND products can be used to capture nucleic acids from cell lysates (Figure 6).

DNA-BIND Thermowell plates can be used for solid-phase PCR enabling quick and effective recovery of PCR products. Assay optimization requires that the guidelines in the Materials and Methods be determined empirically for a spe-

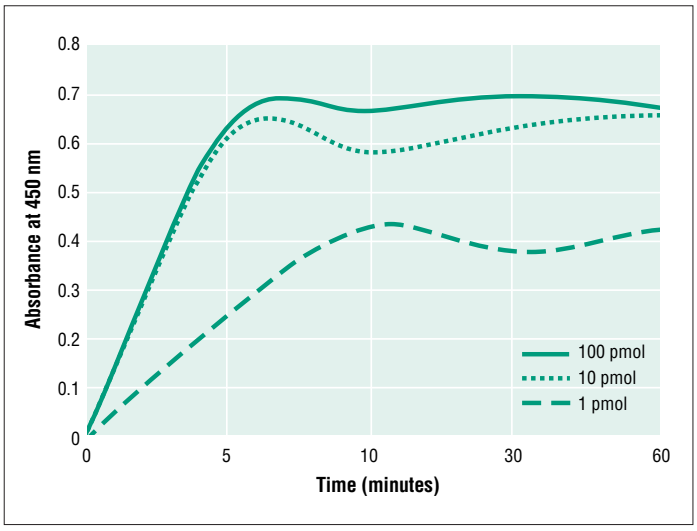

Figure 2. Kinetic analysis of oligonucleotide immobilization. A 30mer with a 5' amine C-12 linker and 3' fluorescein, at concentrations of 1.0, 10, and 100 pmol/100 µL in coupling buffer was incubated in wells of DNA-BIND plates for 0 to 60 minutes. Following washing and blocking, binding was detected spectrophotometrically using an anti-fluorescein HRP-conjugated antibody and TMB substrate.

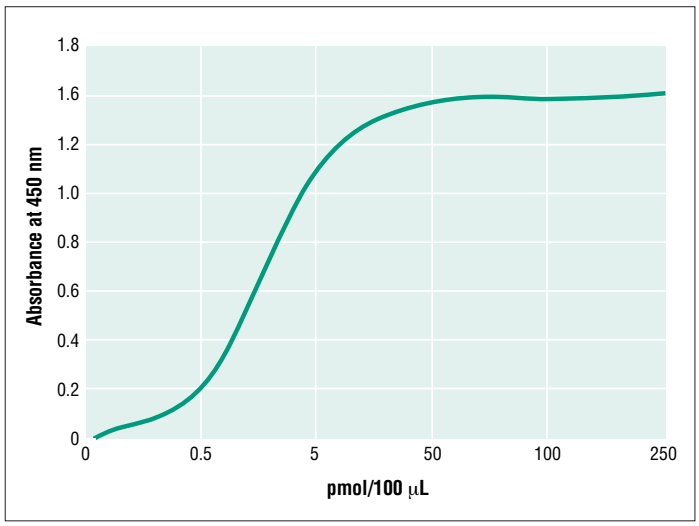

Figure 3. Detection of hybridization at increasing concentrations of oligonucleotide. Following immobilization of increasing concentrations of an aminated oligonucleotide to the DNA-BIND surface, equimolar amounts of a complementary oligonucleotide labeled with a 5' fluorescein molecule, was allowed to hybridize at 55°C for 30 minutes as stated in Materials and Methods.

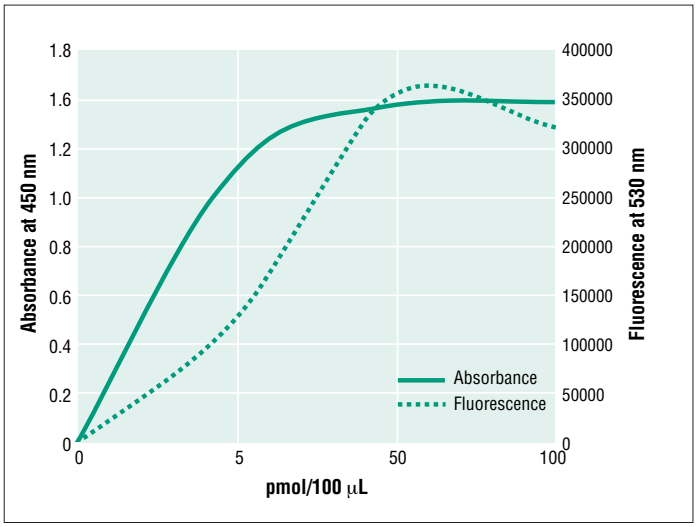

Figure 4. Direct and indirect detection of immobilized oligonucleotides. A 30mer with a 5' amine C-12 linker and 3' fluorescein, at concentrations from 0 ƒ) 100 pmol/100 µL was immobilized in wells of DNA-BIND plates. Following washing and blocking, the immobilized oligonucleotides were first detected by monitoring fluorescence at 530 nm. Subsequent detection occurred spectrophotometrically after incubation with an anti-fluorescein HRP-conjugated antibody as stated in Materials and Methods.

cific assay, but in general, any modifications to optimize a standard PCR reaction will also improve solid-phase PCR. In the liquid phase, a 1:12.5 ratio of forward (capture) primer in solution, to the forward primer in the solid-phase (immobilized), has provided optimal results for this particular set of reaction components (Figures 7a and 7b). This ratio may need adjustment, along with cycling times and temperatures, depending on the reaction components of other systems. Agarose gel analysis of the liquid phase can help diagnose problems with amplification. The concentration of PCR products in the liquid phase will usually be lower than the concentration of product produced in a standard PCR, since much of the product will be detected in the solid-phase.

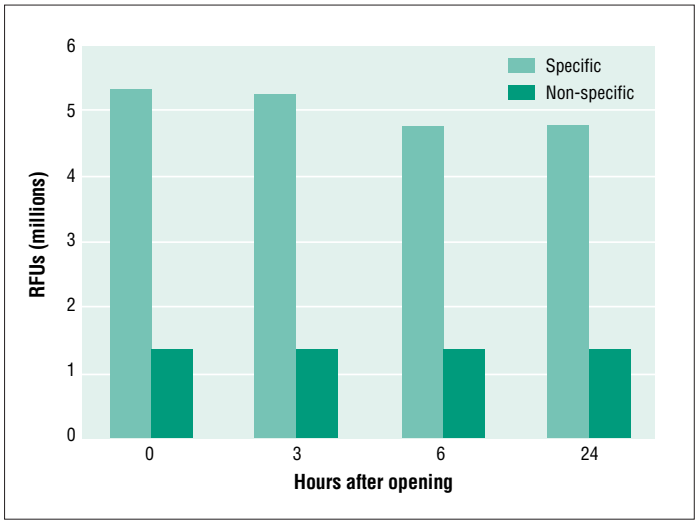

Figure 5. Surface reactivity to oligonucleotide coupling over time. A 30mer with a 5' amine C-12 linker and 3' fluorescein, at a concentration of 50 pmol/100 µL, was incubated in wells of DNA-BIND™ plates that had been removed from packaging, covered with a lid, and left on the benchtop at room temperature for 0 to 24 hours.

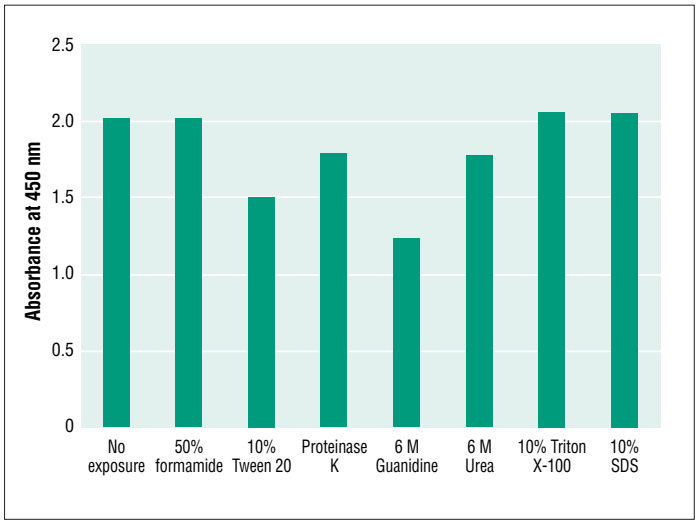

Figure 6. Stability of immobilized oligonucleotides following exposure to cell lysis or denaturing reagents. Following immobilization of 50 pmol/100 µL aminated oligonucleotide to wells of DNA-BIND plates, 100 µL/well of cell lysis or denaturing reagents were added and allowed to incubate for 60 minutes at room temperature. A complementary oligonucleotide labeled with a 5' fluorescein molecule was allowed to hybridize at 55°C for 30 minutes. Detection occurred as stated in Materials and Methods.

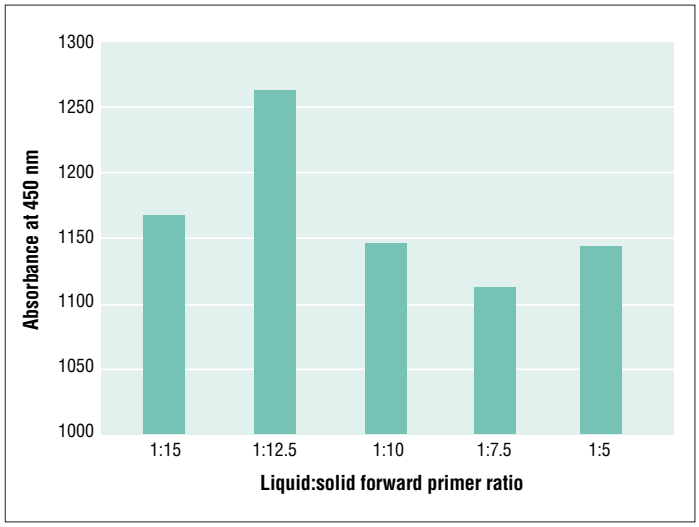

Figure 7a. Solid-phase PCR product. 5' aminated oligonucleotide at 50 pmol/100 µL of coupling buffer was added to wells of DNA-BIND Thermowell plates. Following a 15 minute incubation, the wells were washed and blocked. Ingredients for PCR were added, including concentrations of the coupled oligonucleotide at ratios of 1:5–1:15 liquid-to-solid phase oligonucleotide proportions. PCR was carried out and solid-phase PCR product detected as stated in Materials and Methods.

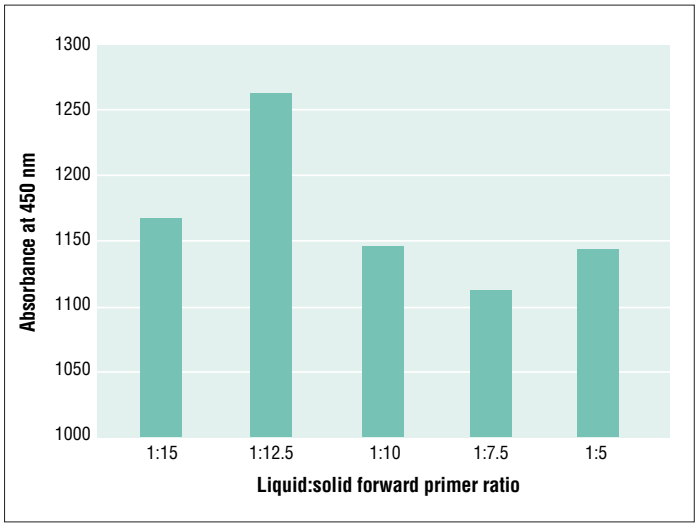

Figure 7b. Liquid-phase PCR product. Following PCR as stated in Figure 7a, liquid-phase PCR product was mixed with sample buffer and electrophoresed through a 1% agarose gel. The gel was soaked in SYBR™ Green 1 Nucleic Acid Stain, and imaged on a FluorImager 595. Bands were quantitated using ImageQuant™ software.

Conclusions

- Corning DNA-BIND™ 96-well plates, 1 x 8 Stripwell™ plates and Thermowell M PCR plates require no activation for covalent immobilization of primary amine containing biomolecules.
- The initial coupling event of amine C-12 modified oligonucleotides with the DNA-BIND™ surface occurs very rapidly.
- Aminated oligonucleotides immobilized on DNA-BIND 96-well plates, 1 x 8 Stripwell plates, and Thermowell® M PCR plates are stable to common cell lysis and denaturing reagents.
- Corning DNA-BIND 96-well plates, 1 x 8 Stripwell plates and Thermowell M PCR plates can facilitate the development of high-throughput solid-phase assays to analyze interactions between numerous biomolecules.

Additional Literature Available Upon Request

1. Comprehensive DNA Probe Assay Bibliography, Ordering code MB-102.
2. Technical Note: "Understanding and Working with Nucleic Acids," Ordering code MB-103.
3. DNA-BIND Application Guide (revised), Ordering code MB-101.

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