EFFECTS OF PROBE LENGTH AND SECONDARY STRUCTURE ON DNA MICROARRAY HYBRIDIZATIONS: IMPLICATIONS FOR DESIGN OF PATHOGEN DETECTION ASSAYS

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ABSTRACT

A number of investigators have proposed using DNA microarrays as part of pathogen detection assays. False negative hybridizations can occur if the target DNA hybridizes to itself and other target DNA, thus inhibiting hybridization to complementary microarray probes. We hypothesized that longer probes can compete with this 'secondary structure' better than shorter probes. To test this we constructed a prototype microarray using a series of short and long probes that were specific to the 16S ribosomal DNA gene of Escherichia coli. Three different targets (PCR un-nick translated, PCR nick translated, and nick translated genomic DNA) were hybridized to an array and assessed by a Tyramide Signal Amplification™ biotin system. Our results verified that hybridization of short probes to target sequences is greatly affected by secondary structure, while long probes provide enhanced signal intensities. Using long probes can offer three specific advantages to microarray design: 1) mitigation of secondary structure, 2) enhanced analytical sensitivity, and 3) greater tolerance to base pair mismatches.

INTRODUCTION

Several investigators have proposed using DNA microarrays as part of pathogen detection systems.[2] The most common strategy has been to use PCR to amplify pathogen-specific DNA sequences that are subsequently hybridized to a microarray. PCR amplicons ("targets") anneal to complementary "probe" sequences that are affixed to a solid substrate (glass or silicon wafer). The target probe duplexes are then detected using a fluorescence system that utilizes reporter molecules such as fluorophores, chemiluminescent moieties, or radioactive isotopes.

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[2] See Call et al., 2001b; Chandler et al., 2001; Chizhikov et al., 2001; Chizhikov et al., 2002; González et al., 2004; Keramas et al., 2004; Kingsley et al., 2002; Lemarchand et al., 2004; Panicker et al., 2004; Staub et al., 2002; Volokhov et al., 2002; Wang et al., 2002; Warsen et al., 2004; Wilson et al., 2002; and Zhou 2003.

Microarray probes are typically oligonucleotides (9- to 70-mer) or PCR products (>150 bp), both of which present unique tradeoffs in detector performance. An important relationship to consider is that under "standard" hybridization conditions, probes and targets need ≥ 85% sequence similarity to properly anneal (see Call et al., 2001b; Letowski et al., 2004; and, Wu et al., 2001). Thus, short oligonucleotide probes (e.g. 20-mer) can be highly specific for 1-2 base-mismatches, whereas longer probes (150-1,500 bp) can tolerate many more base-mismatches. While sensitivity to 1-2 base-mismatches is useful for subtyping, we argue that having tolerance for more mismatches is an important property. This is because naturally occurring genetic variants will still hybridize to the probes when the total number of base-mismatches does not exceed 15 percent of the total probe sequence (Letowski et al., 2004). Long probes are less likely to produce false negatives compared to short probes. Another significant property of long probes is their increased analytical sensitivity. That is, longer probes are more sensitive to dilute target concentrations, thus making them more sensitive detectors (Letowski et al., 2004; Stillman and Tonkinson, 2001; and Wu et al., 2001).

While longer probes offer greater sensitivity and flexible detection, they may provide another important benefit: mitigating secondary structures. Secondary structures form when a single-stranded target folds and anneals to itself and other targets. In some situations, these structures prevent target interaction with the microarray probe (Chandler et al., 2003; Lane et al., 2004). This is distinguished by observing the appropriate PCR amplicons on gel electrophoresis but failing to observe hybridization of these PCR amplicon targets to a complementary microarray probe. Secondary structure has been hypothesized as a mechanism responsible for failed microarray hybridizations with 16S rDNA probes. Lane et al. (2004) demonstrated that hybridization failure is haphazard and uncorrelated with target length. When targets were fragmented by nick translation, they displayed successful hybridization of all probes to the appropriate target. This is consistent with disruption of secondary structure, thereby permitting target sequences to anneal with appropriate probes.

Although nick translation seems to alleviate secondary structure, this process comes with added expense and assay time. Small et al. (2001), Peplies et al. (2003), and Chandler et al. (2003) introduced sequence-specific helper oligonucleotides to facilitate relaxation of secondary structure. This technique used short oligonucleotides located upstream of the target region to relieve secondary structure. This allowed the microarray probe to bind to the target region, which was otherwise unavailable due to secondary structure. Unfortunately, this strategy is difficult to generalize for many target sequences, and it may be sensitive to natural genetic variation leading to an elevated number of false negatives. Peplies et al. (2003) also found that adding Poly (A) spacers to the probe sequences relieved secondary structures.

In this study we tested the hypothesis that longer probes successfully mitigate secondary structure. If correct, we argue that long probes are best suited for microarray pathogen detectors because of: 1) an ability to mitigate secondary structure in conjunction with reducing assay costs; 2) an increased analytical sensitivity; and, 3) an increased tolerance for naturally occurring genetic diversity.

METHODS

Primers and Probes

Eight sense and one antisense oligonucleotide sequences from Lane et al. (2004) and seven additional antisense oligonucleotide sequences were used as PCR primers to generate probes of variable length from the 16S rDNA gene in *E. coli* K12 (Genbank reference #AE000542). In this case one sense oligonucleotide, 16s_fwd001, was paired against one of seven different antisense oligonucleotides ("rvscomp" and 16s1518NR) in a PCR reaction to produce probes ranging from 245 to 1518 bp in length (see Appendix: Table 1, Set I; Fig. 1a). Eight additional PCR amplicons

(162-223 bp) were generated using a combination of eight sense primers against seven antisense rvscomp primers (Set II, Table 1; Fig. 1a). In addition to the PCR probes, eight 22-mer oligonucleotide probes (Table 2; Fig. 1b) from Lane et al (2004) were added to the array as well as three additional oligonucleotides (35-mer, 50-mer, and 70-mer) that were designed using commercial software (Vector NTI, version 9.0, Informax, Bethesda, MD) (see Table 2).

Microarray Preparation

Teflon-masked, twelve-well glass slides (Erie Scientific, Portsmouth, NH) were prepared with epoxy-silane (3-Glycidoxypropyltrimethoxysilane) prior to probe printing (Call, Chandler and Brockman, 2001a). Briefly, slides were sonicated with 2.5% 70 contrad detergent (Fisher Scientific) for 2 min and rinsed in distilled water. Dried slides were then incubated in 3N HCl for 1 hr, rinsed and dried. Slides were incubated in 2% epoxy-silane, 98% methanol for 15 min, then rinsed twice with 100% methanol and dried with compressed air.

Oligonucleotides were diluted to 60 µM in 1X print buffer (0.2 M Na_2HPO_4, 0.4 m NaCl, 0.01% SDS), pH 11, and arranged in a 96-well tray (Robbins Scientific Corporation, 2002) with 15 µl per well. PCR probes were prepared by ethanol precipitating the products, and diluting to 75 ng/µl in 1X print buffer. Oligonucleotides and PCR probes were printed in quadruplicate onto prepared slides using a Microgrid II Arrayer (Genomic Solutions, Ann Arbor, MI). Arbitrary 25-mer biotinylated oligonucleotides were printed on the slides as positive controls for detection chemistry.

Target Preparation

Three different targets were used in these experiments: PCR amplicons, nick translated PCR amplicons, and nick translated genomic DNA. Template for PCR reactions and for genomic DNA hybridizations was prepared from an overnight culture (3 ml) of *E. coli* K-12 using a commercial kit (DNeasy tissue kit, Qiagen, Valencia, CA). The PCR amplicons consisted of a 1,633 bp fragment generated by pairing the pre_fwd001 primer against the 16s1568R primer, where both primers were conjugated with biotin (Table 1). Each PCR reaction included: 1X PCR buffer (Fisher Scientific), 1.5 mM $MgCl_2$, 200 µM each dNTPs, 200 nM of each primer, 0.6-1.25 units Taq, and 30 ng of genomic DNA. Thermalcycling conditions included 95˚C for 5 min, followed by 30 cycles of 95˚C for 30s, 62˚C for 45s, and 72˚C for 60s; a final extension of 72˚C for 10 min completed the reaction. PCR amplicons were verified using 1% agarose gel electrophoresis.

Nick translated PCR amplicons (1,633 bp) were prepared by ethanol precipitating the finished PCR reaction and nick translating the amplicons using a commercial kit (Bionick, Invitrogen Corp). This kit incorporates biotinylated dATPs into the nick translated products. After 2 hrs of incubation (16˚C), products were ethanol precipitated and resuspended in 60 µl of microarray hybridization buffer (4X SSC, 5X Denhardt's solution). The third target consisted of nick translated genomic DNA. The same nick translation protocol was used except 1 µg of genomic DNA was substituted for the ethanol precipitated PCR amplicons.

Hybridization and Detection

Slides were incubated in TNB blocking buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.5% blocking reagent [supplied in Tyramide Signal Amplification™ biotin system; TSA™ kit; Perkin-Elmer, Boston MA]) for 30 min at room temperature. A total of 21.4 µl PCR amplicons (either un-nick and nick translated) was diluted with 38.6 µl water, and 50.0 µl 2X hybridization buffer (8X SSC, 10X Denhardt's Solution). This mixture was then serially diluted from 1:1 to 1:150 for hybridization. Genomic DNA was initially diluted 1:5 with 1X hybridization buffer and then serially diluted to 1:640 for hybridization. This DNA was denatured at 95˚C for 2 min and

immediately incubated on ice until distributed to the microarray slide (35 µl per masked well). The microarray was then incubated over night (or a minimum of 4 h) in a 50 ml conical tube with damp filter paper (55˚C).

After hybridization, slides were washed in triplicate with TNT buffer (0.1 M Tris-HCl, 1.5 M NaCl, 0.05% Tween 20™, deionized water). Wells were incubated with streptavidin horseradish-peroxidase in TNB for 30 min. After rinsing three times in TNT, a solution of 10% fetal equine serum (FES; Sigma Chemical Company; St. Louis, MO) in 2X SSC was incubated on each well for 30 min, then rinsed. Biotinyl-tyramide (10% ,TSA™ kit) in 1X Amplification Diluent (TSA™ kit) was applied to the wells for 10 min and rinsed. Finally, streptavidin conjugated to Alexa Fluor 546 (Mol. Probes, Eugene, OR) was diluted 1:500 (1X SSC, 5X Denhardt's), and incubated in each well for 1 hour. Slides were then rinsed and dried using a micorarray centrifuge (ArrayIt, Sunnyvale, CA). An *ArrayWorx*^{e}™ (Applied Precision, Issaquah, WA) scanner was used to image each slide, while *SoftWorx*™ (Applied Precision) was used to quantify results. Images were scanned at 10 µm pixel resolution and stored as 16-bit TIF files (pixel values 0-65,535). Median pixel values for each replicate probe spot were output and data were managed using *Microsoft*™ *Access* and *Excel* (Microsoft Corp., Redmond, WA). All experiments were replicated three times and standard errors calculated from three independent experiments.

RESULTS AND DISCUSSION

Lane et al (2004) designed an array composed of eight 22-mer oligonucleotide probes that were systematically distributed across the *E. coli* 16S rDNA gene (Fig. 1b). They then amplified eight fragments of different length from this gene and demonstrated inconsistent hybridization results, corresponding to interference from secondary structure. In the present study we used the same 22-mer oligonucleotide probes in addition to eight PCR amplified fragments (245-1518 bp; Set I) and eight shorter PCR probes (162-223 bp; Set II) distributed throughout the gene (Figs. 1a and 2). Three additional "long" oligonucleotides (35-mer, 50-mer, and 70-mer) were included within the 5-prime region of the 16S rDNA gene (Fig. 1b) where our experience suggests that secondary structure has the greatest negative impact on hybridization of this gene. Our study used only one target region (1,633 bp) amplified using primers Pre_fwd001 and 16s1568R (Table 1). The biotinylated primers for this target were positioned outside of all probe regions to avoid primer-probe interactions on the microarray. The resulting PCR amplicons were either unmodified or nick-translated. A third target, genomic DNA, was also nick translated and used for hybridization.

Oligonucleotide Probes

For ease of communication, we designated probes as "5-prime-oligos" (probes 17-20), "3-prime-oligos" (probes 21-24) or "Long-oligos" (probes 25-27) (Tables 1, 2; Fig. 2). Un-nick translated PCR targets hybridized with near saturation signal to the 3-prime-oligos, but failed to hybridize to most of the 5-prime-oligos and all of the Long-oligos (Fig. 3a). Unlike Lane et al (2004), nick translating the target did not mitigate effects of secondary structure for all of the original 5-prime-oligos (Fig. 3a). We re-printed the microarray three times to rule out the possibility that our failure to fully replicate the earlier work was due to improperly printed microarrays. We can only speculate that some other procedure (e.g. labeling or precipitation) affected our ability to hybridize the target to the 5-prime-oligos. Nevertheless, nick-translated PCR products were easily detected using the Long-oligos, whereas these probes were unable to hybridize to the un-nick translated amplicons (Fig. 3a). This result is consistent with relaxed secondary structure resulting from nick translation. With the exception of the 70-mer probe, none of the oligonucleotides detected nick translated genomic DNA, thereby verifying the low analytical sensitivity of shorter probes (Letowski, Brousseau, and Masson, 2004).

PCR Amplicon Probes

The first 16 probes on the microarray were generated by PCR amplification. PCR probes 1-8 (Set I, Table 1; Fig. 1a) ranged from 245 bp to 1518 bp, while PCR probes 9-16 (Set II, Table 1; Fig. 1a) ranged from 162 bp to 223 bp. The detection ability of these probes was indisputably sufficient for all three targets (Fig. 3b). Consistent with our hypothesis, it appears that even moderately long PCR probes (>150 bp) are efficient at competing with target secondary structure. Using these probes, there was no significant difference in hybridization intensities between a nicked PCR target and an un-nicked PCR target (Fig. 3b). Consequently, microarrays designed with moderately long probes (>150 bp) would not require nick translation or other fragmentation schemes for efficient target hybridization.

Analytical Sensitivity

A dilution series of each target was generated to assess the analytical sensitivity of the probes. As expected, the 5-prime-oligos and Long-oligos produced virtually no signal for any dilution for un-nick translated PCR amplicons (Fig. 4a). The 3-prime-oligos achieved saturated signals for the fifth and sixth dilutions (1:5 and 1:1), but decreased rapidly thereafter (Fig. 4a). PCR amplicon, both nick and un-nick translated, were completely saturated across all 6 dilutions when hybridized to PCR probes (Fig. 4a and 4b). Detection of the nick translated PCR amplicons by the 3-prime-oligos and Long-oligos produced saturated intensities for dilutions 5 and 6, but declined in intensity for accompanying dilutions (Fig. 4b). Detection intensities for nick translated gDNA targets decreased after the fourth dilution (1:40) with PCR probes whereas genomic DNA targets were completely undetected by the oligonucleotides (Fig. 4c).

Thus for nick translated PCR amplicons, oligonucleotide probes lost detection ability between the third dilution (1:50) and fourth dilution (1:25), while PCR probes maintained saturated signals across all dilutions for both nick and un-nick translated PCR amplicons. If we consider a signal intensity of 10,000 to be the threshold for positive detection, then based on the genomic DNA dilutions the Set I PCR probes were capable of detecting a 1:320 dilution, equivalent to $7x10^5$ genomes.

Implications for Assay Designs Involving Pathogen Detection

The primary advantage of short oligonucleotide probes is ease of production. An investigator only needs sequence information (e.g., from GenBank) to design synthetic probes, whereas probes produced by PCR initially require gene specific primers and a suitable template to generate the probe. We argue that the production advantage of short probes is outweighed by the significant advantages offered by long probes (>150 bp). As demonstrated here, long probes can successfully mitigate secondary structure that can interfere with hybridization to microarray probes. Although Lane et al. (2004) demonstrated the usefulness of nick translation in alleviating secondary structure, this procedure is expensive and time consuming which may be true for other fragmentation strategies. Furthermore, our study revealed nick translation does not completely mitigate for secondary structure. Addition of sequence specific "helper" or "chaperone" oligonucleotides also help mitigate secondary structure (Chandler et al., 2003; Peplies, Glockner and Amann, 2003; Small et al., 2001), but these procedures are difficult to generalize for larger microarrays.

The second advantage to long probe sequences is the promotion of increased analytical sensitivity. As confirmed here (Fig. 4) and by others (Letowski, Brousseau, and Masson, 2004; Stillman and Tonkinson, 2001; Wu, et al., 2001), dilute target sequences are better detected with longer probes. The third advantage to long microarray probes is elevated tolerance of base

mismatching. Different pathogen strains may be slightly different from one another due to natural genetic variation; under these circumstances, a long probe is still able to hybridize the correct target homologue whereas a short probe will be less "tolerant" to unexpected genetic variation. All three of these advantages contribute to a reduced probability of false negative detection events, which is a critical concern in pathogen detection assays.

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APPENDIX: Tables and Figures

Table 1. PCR primers used in this study.

Sequence A	Sequence B	Sequence (5'—3')	Bp	Probe name (#)
Set I				
16s_fwd001		AATTGAAGAGTTTGATCATGGCTCA		
	rvscomp224	TAATCCCATCTGGGCACATCTG	245	PCR 245 (1)
	rvscomp442	AACTTTACTCCCTTCCTCCCCG	462	PCR 462 (2)
	rvscomp618	TCAGATGCAGTTCCCAGGTTGA	639	PCR 639 (3)
	rvscomp798	ATCGTTTACGGCGTGGACTACC	819	PCR 819 (4)
	rvscomp974	GGATGTCAAGACCAGGTAAGGTTC	997	PCR 997 (5)
	rvscomp1166	ATCCCCACCTTCCTCCAGTTTA	1187	PCR 1187 (6)
	rvscomp1356	CGTATTCACCGTGGCATTCTGA	1377	PCR 1377 (7)
	EubA1518NR	AAGGAGGTGATCCANCCRCA	1518	PCR 1518 (8)
Set II				
rvscomp224	16s_fwd001	AATTGAAGAGTTTGATCATGGCTCA	223	PCR 001 (9)
rvscomp442	16s_fwd224	CAGATGTGCCCAGATGGGATTA	218	PCR 224 (10)
rvscomp618	16s_fwd442	CGGGGAGGAAGGGAGTAAAGTT	176	PCR 442 (11)
rvscomp798	16s_fwd618	TCAACCTGGGAACTGCATCTGA	180	PCR 618 (12)
rvscomp974	16s_fwd798	GGTAGTCCACGCCGTAAACGAT	176	PCR 798 (13)
rvscomp1166	16s_fwd974	GAACCTTACCTGGTCTTGACATCC	192	PCR 974 (14)
rvscomp1356	16s_fwd1166	TAAACTGGAGGAAGGTGGGGAT	190	PCR 1166 (15)
EubA1518NR	16s_fwd1356	TCAGAATGCCACGGTGAATACG	162	PCR 1356 (16)
Other				
Pre_fwd001	16s1568	TACCAAGTCTCAAGAGTGAACACG TCAGAATGCCACGGTGAATACG	1,633	Amplicon target used for hybridization studies

Note: PCR probes were generated by pairing the primer in sequence A column with the primer in sequence B column. Numbers in parentheses are individual probe names as referenced in the text and Fig. 2 and 3b. Primers pre_fwd001 and 16S1568 were conjugated with biotin

Table 2. Oligonucleotide probes used in this study.

Probe Name	Probe Sequence (5'—3')	Size (nt)
5-prime-oligos		
16s_hyb083 (17)	TTGCTGTTTCGCTGACGAGTGG	22
16s_hyb275 (18)	GCGACGATCCCTAGCTGGTCTG	22
16s_hyb489 (19)	CGCAGAAGAAGCACCGGCTAAC	22
16s_hyb736 (20)	CCCTGGACGAAGACTGACGCTC	22
3-prime-oligos		
16s_hyb933 (21)	ACAAGCGGTGGAGCATGTGGTT	22
16s_hyb1136 (22)	GGCCGGGAACTCAAAGGAGACT	22
16s_hyb1205 (23)	GCCCTTACGACCAGGGCTACAC	22
16s_hyb1452 (24)	GGAGGGCGCTTACCACTTTGT	22
Long oligos		
35Hyb471 (25)	TTTGCTCATTGACGTTACCCGCAGAAGAAGCACCG	35
50Hyb473 (26)	TGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGC	50
70Hyb451 (27)	AAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCA	70

Note: Probe names include a numeric designation. Probes 17-24 were originally published by Lane et al. (2004).

Figure 1. Probe Derivations

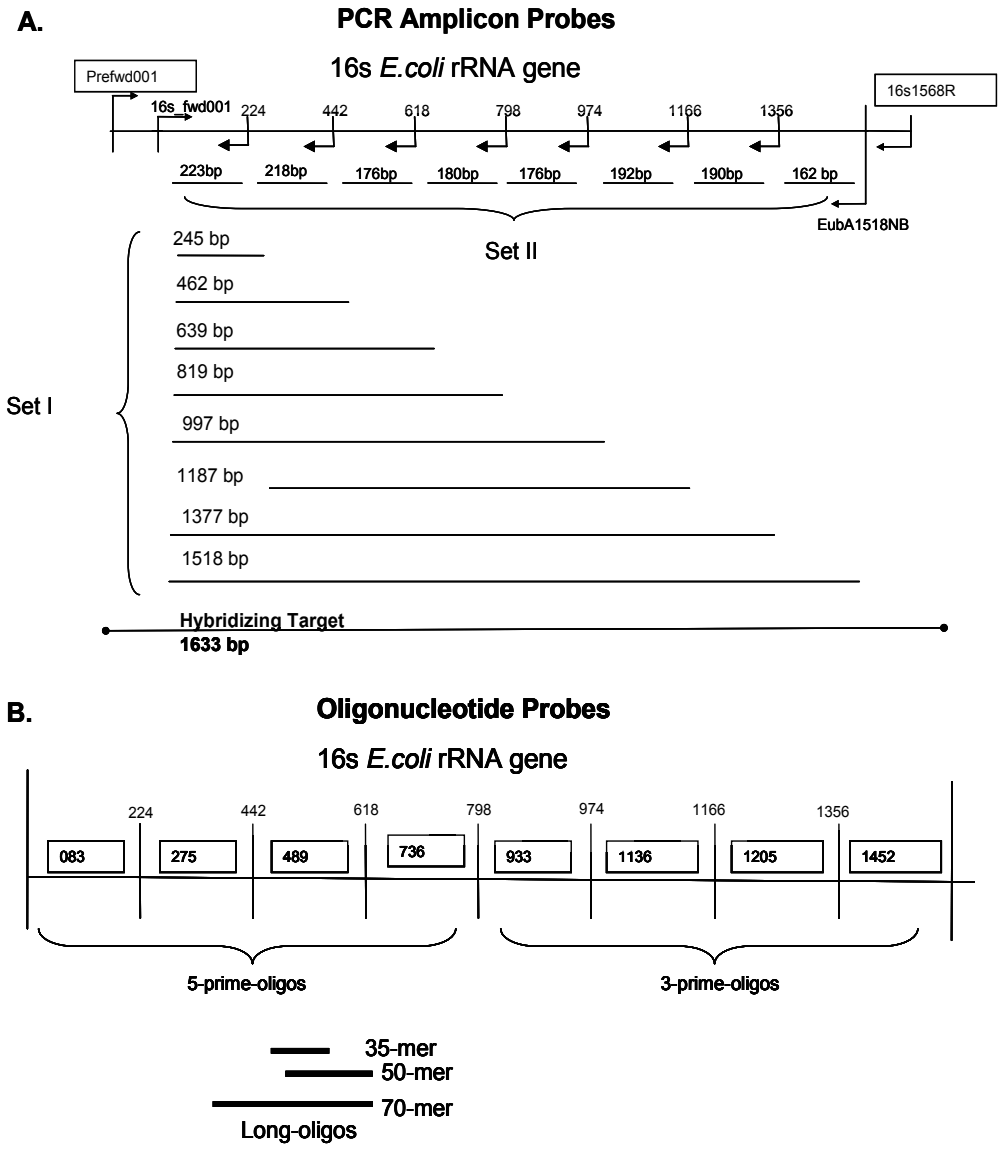
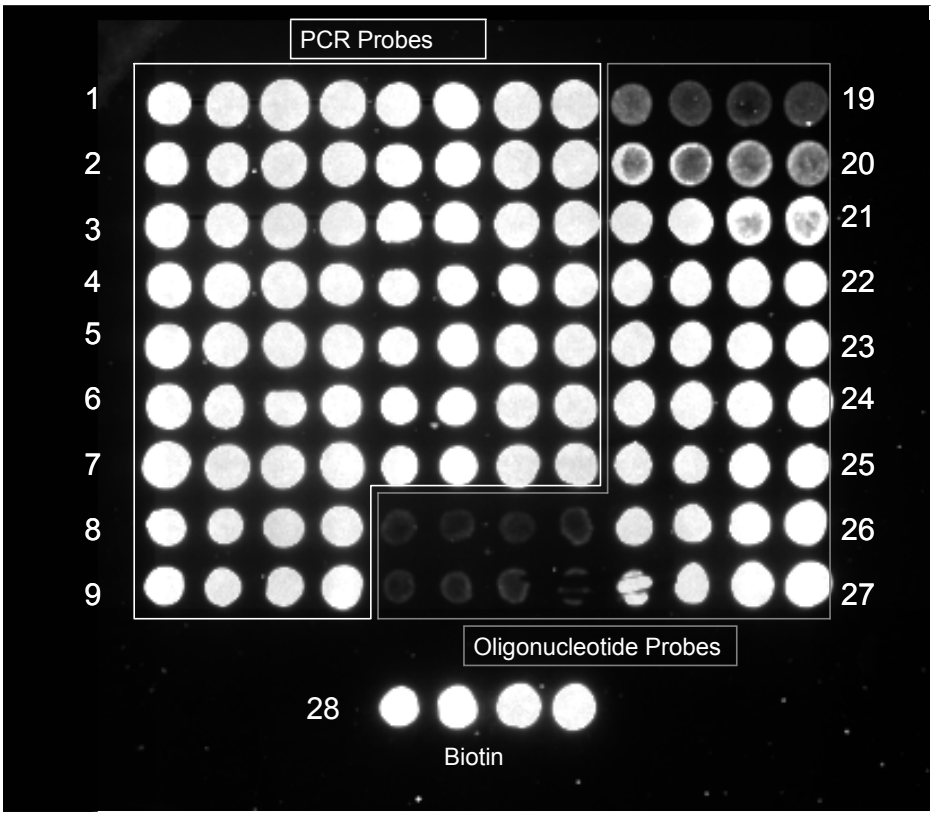


Figure Legend: (A) PCR probes were generated from eight antisense primers (rvscomp224-rvscomp1356) against one sense primer (16s_fwd001) (Set I). An additional set of PCR probes (Set II), approximately 200 bp each, were generated as described in Table 1. (B) 5-prime-oligos and 3-prime-oligos were utilized from Lane et al. (2004), and Long-oligos were designed for the present study. Numbers in boxes illustrate oligonucleotide positions relative to primers identified in Fig. 1A. The Long-oligos are depicted as the actual locations relative to the corresponding primers (between primers 224 and 618).

Figure 2. Microarray Probe Layout



Probe Order

1. PCR 1518	10. PCR 224	19. 16s_hyb489
2. PCR 245	11. PCR 442	20. 16s_hyb736
3 PCR 462	12. PCR 618	21. 16s_hyb933
4. PCR 639	13. PCR 798	22. 16s_hyb1136
5. PCR 819	14. PCR 974	23. 16s_hyb1205
6. PCR 997	15. PCR 1166	24. 16s_hyb1452
7. PCR 1187	16. PCR 1356	25. 35Hyb471
8. PCR 1377	17. 16s_hyb083	26. 50Hyb471
9. PCR 001	18. 16s_hyb275	27. 70Hyb451

28. Biotin control

Figure Legend: Probe layout for 12-well, Teflon-masked, microarray slides. Each well encompassed one microarray as shown here. Probes were spotted in quadruplicate from left to right, constituting three unique columns of probes. Probes are numbered as shown.

Figure 3. Target Comparison to Each Probe

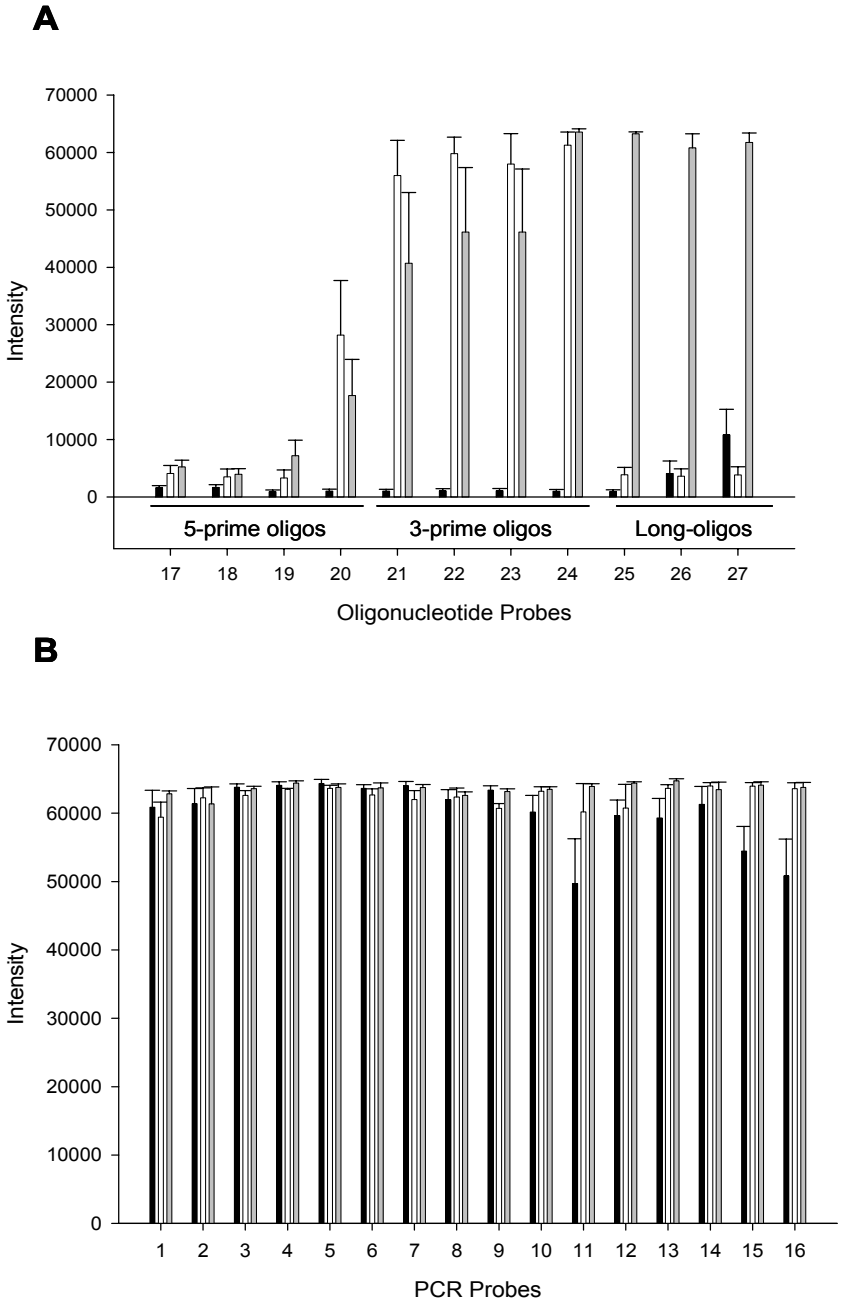


Figure Legend: (A) Signal intensities for all PCR probes hybridized to each of three targets at a 1:50 dilution (solid bars = unmodified PCR amplicons, open bars = nick translated PCR amplicons, and grey bars = genomic DNA). (B) Signal intensities for oligonucleotide probes hybridized to each target at a 1:40 dilution. Error bars=standard error.

Figure 4. Target Dilution Series Comparison

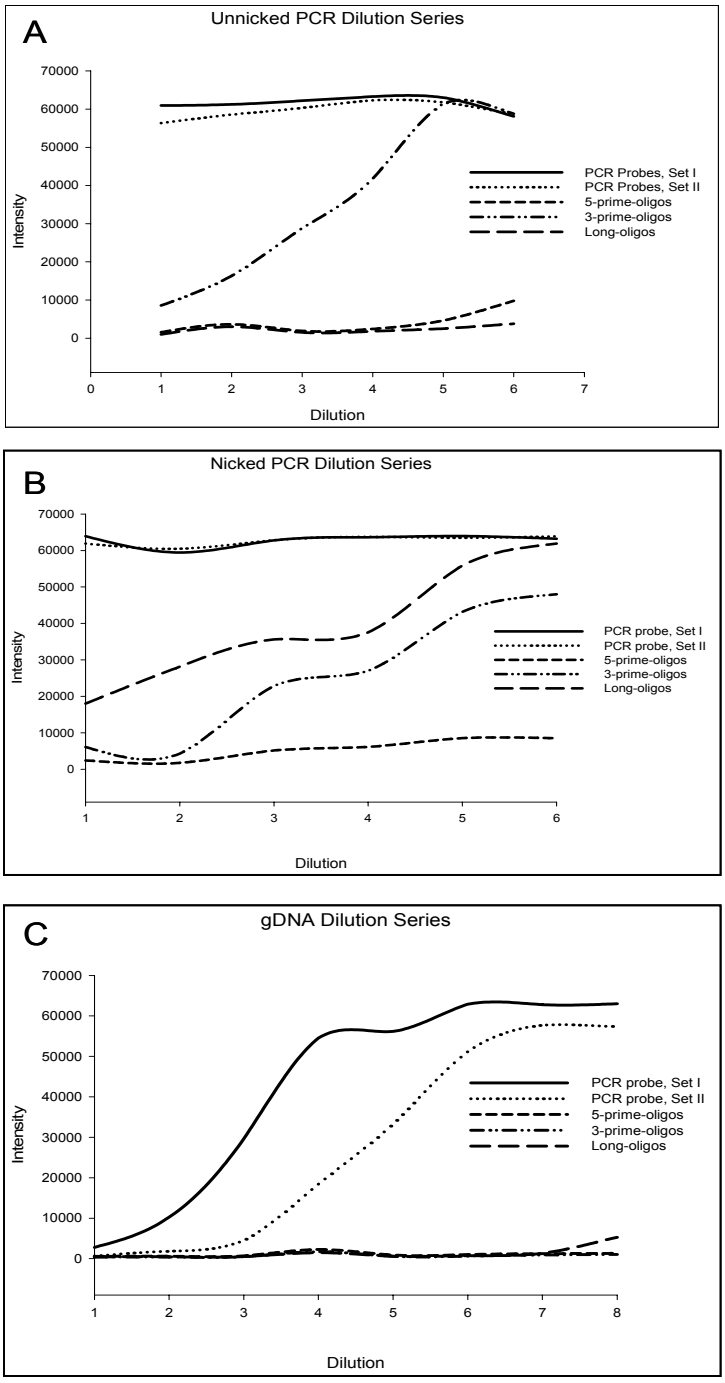


Figure Legend: (A) and (B): Dilutions for PCR targets were labeled 1-6 and are as follows: 1:150, 1:100, 1:50, 1:25, 1:5, and 1:1. (B) Dilutions for genomic DNA were labeled 1-8 which included: 1:640, 1:320, 1:160, 1:80, 1:40, 1:20 1:10, and 1:5.