Amplicon secondary structure prevents target hybridization to oligonucleotide microarrays

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We have read the Guidelines for Mentoring Honors Students, and we agree to serve as faculty thesis

advisor for Samantha Lane.

The preliminary topic for the thesis is: Use of DNA microarrays as end-point detectors for pathogens.

and the central question is: How does amplicon secondary structure impact microarray hybridization?

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Precis

DNA microarrays are a revolutionary tool that permits simultaneous detection of thousands of genes. Microarrays are most often used for analysis of gene expression, but they are also used for studies in comparative genomics, and increasingly, for pathogen detection. This latter application is the focus of my thesis.

DNA microarrays consist of gene-specific DNA fragments (called “probes”) that are typically bound to a glass slide. When complementary genes (called a “targets”) are applied to the slide, the targets will anneal to their respective probes. The probe:target duplex is then detected using a method such as fluorescence. In this study, I employed a combination of biotin and streptavidin chemistry with enzymatic amplification to detect the probe:target duplex.

When microarrays are used for pathogen detection, they are typically coupled with polymerase chain reaction (PCR). In this format, PCR is used to amplify many copies of a gene and these copies are then hybridized to the microarray to determine which sequence is present. While this method is quite suitable for detecting a single copy of a gene, we have encountered a recurring problem whereby known genes are amplified, but fail to hybridize to their respective probes.

The unpredictable nature of these failures was particularly interesting to me because: (1) work in this area would provide me with a better understanding of the technology; and (2) I hoped that my findings would provide some guidelines for improving assay design and implementation. Ultimately, I wanted my research to aid development of rapid accurate detection of potentially dangerous pathogens in food production as well as wildlife.

The unpredictable nature of hybridization failure led me to the hypothesis that target secondary structure might be interfering with target hybridization to the microarray. Secondary

structure occurs when DNA strands self-anneal and form complex 3-dimensional structures that "hide" complementary sequences from the microarray probes. I tested this hypothesis by designing a simple micorarray suitable for detecting eight regions of the *Escherichia coli* 16S rDNA gene. Eight DNA sequences of varying lengths (184-1,517 bp) were amplified using PCR and labeled with a single terminal biotin (5-prime terminus). These sequences (called "amplicons") were then hybridized to the microarray and detected as described. I found that some, but not all of the probes hybridized to their respective amplicons. Hybridization bias was tested by moving the single biotin to the 3-prime terminus. This manipulation mirrored the same hybridization failures. The amplicons were then nick-translated. This process breaks the amplicon into smaller biotinylated fragments and in theory, should eliminate any secondary structure. In fact, the nick-translated products hybridized to all of the probes thereby indicating that nick-translation eliminated any detrimental effects of secondary structures that would have otherwise prevented accurate hybridization. Despite the increased cost of nick-translating each amplicon, I concluded that this strategy not only decreases time investment into the design of a new assay, it also will aid in the reduction of false negatives that might arise from secondary structures arising due to novel sequence mutations.

By studying the applications and possible underlying problems to this detection system, I was able to gain detailed insights regarding this system. The findings were sufficiently important that I also gained valuable experience through submission of an earlier draft of this thesis for review for publication in a peer-reviewed journal (Biosensors and Bioelectronics). The paper was formally accepted for publication in Feb. 2004.

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1. Introduction

Planar microarrays generally consist of 10 to >10,000 probes that are complementary to genes or specific sequences of interest to the investigator. The probes, which are composed of either oligonucleotides (9-70 mer) or PCR products (300-2,000 bp), are spatially registered in a lattice pattern. Labeled targets (cDNA, mRNA, tRNA, aRNA, PCR amplicons, genomic DNA, plasmid DNA) are incubated on the array after which hybridized products are typically detected by fluorescence. Target labeling generally involves direct incorporation of fluorophore conjugated nucleotides or incorporation of other "reporter molecules" such as biotin for indirect detection (i.e., streptavidin-biotin chemistry) or amplification (e.g., tyramide signal amplification or dendrimers).

DNA microarrays are most recognized for their use in studies of gene expression involving hundreds to thousands of genes. By analyzing gene expression, clues to the function of specific genes can be identified, aiding in the development of vaccines and therapeutic intervention (Debouck and Goodfellow, 1999; Rubin and Merchant, 2000; Yoshioka et al., 2000). DNA microarrays have also been used in DNA sequence analysis (Pease et al., 1994), and for comparative genomics, whereby genetic content of multiple strains of bacteria can be compared (Borucki et al., 2003; Call et al., 2003a; Cho and Tiedje, 2001). DNA microarrays can also be coupled with polymerase chain reaction (PCR), in which case the microarray can be used as an "end-point" detector that is potentially suitable for sensitive and simultaneous detection or characterization of multiple pathogens (Call et al., 2001a; Call et al., 2003b; Chizhikov et al., 2001; Chizhikov et al., 2002; Straub et al., 2002; Volokhov et al., 2002; Wang et al., 2002). This latter application is the focus of this paper.

A conventional format for an end-point detector is to design sequence specific oligonucleotides (15-25 mer) that are then deposited on a solid substrate (Guo et al., 1994), or that are synthesized *in situ* (Wilson, et al., 2002). The oligonucleotide probes are designed using various commercial or freeware algorithms that check for dimers, hairpin loops, and normalized melting temperatures. Nevertheless, even with careful consideration of probe design and hybridization conditions, we have encountered a number of probe failures whereby a target has not hybridized to a probe as expected. The question is, if the majority of these failures are due to design flaws with the oligonucleotide sequences themselves, or as suggested by Chandler et al. (2003), are these failures due to target secondary structure? If failures are due to structural features of the targets themselves, then it is possible that these features could also generate false negatives during real-world applications such as pathogen detection. Consequently, answers to these questions would aid in the design and implementation of microarray based assays; preferably without compromising sensitivity, specificity, and total cost. In this paper, we compare microarray hybridizations using terminally labeled PCR products (amplicons) and nick translated amplicons to determine how target secondary structure and labeling system impact hybridization and assay sensitivity. We show that target secondary structure can be a significant limitation, but that there is a relatively simple means to correct for this problem.

2. Materials and Method

2.1. 16s rDNA primer and probe sequences

A 1,540 bp region of the *E. coli* 16s rDNA gene was chosen for primer and probe design using commercial software (Primer Premier, version 5.0, Premier Biosoft International, Palo Alto, CA). A series of unmodified, sense primers (n= 8) were designed to PCR amplify specific

regions of the 16s rDNA gene in conjunction with the EubA1518R antisense primer (Table 1). The EubA1518R primer included a biotin moiety conjugated to the 5′ terminus. These eight sense primers were staggered *ca*. 200 bp apart to produce amplicons of varying lengths (162-1,517 bp) (Fig. 1). Unmodified oligonucleotide probes (25-mer; Invitrogen Corp., Carlsbad, CA), were designed to hybridize to the regions located between sense primers. Oligonucleotides were reconstituted in 1X TE (10 mM Tris-base pH 8, 1 mM EDTA pH 8, deionized water), followed by quantification using a biophotometer (Eppendorf Scientific, Westburg, NY) and dilution to 60 μM in 1X print buffer (0.1 M Na_2HPO_4, 0.2 M NaCl, 0.01% SDS).

2.2 Microarray construction

Microarrays were constructed following the methods of Call et al. (Call et al., 2001b) with minor modifications. Briefly, 10-well, Teflon®-masked slides (Erie Scientific, Portsmouth, NH) were sonicated in a 2.5% Contrad 70 detergent (Fisher Scientific, Pittsburgh, PA) for 2 min. Slides were then rinsed three times in deionized water and dried with compressed air followed by 1 h incubation in 3N HCl. Cleaned slides were then rinsed three times in deionized water, dried, derivatised with 2% 3-Glycidoxypropylmethoxysilane (epoxy-silane; Sigma Aldrich, St. Louis, MO) in methanol for 15 min, and rinsed in deionized water. Reconstituted probes were printed onto wells of the Teflon®-masked slides in replicates of four spots using a MicroGrid II Arrayer (BioRobotics; Woburn, MA). Arbitrary 25-mer, biotinylated oligonucleotides (5 μM) were included with every microarray as positive controls for detection chemistry and to assist in orienting array images. The printing protocol involved repeated wash baths to clean the pins between probes (4 pins, 4 sec, 2 times) with a 0.5 sec flush and 6 sec vacuum dry to reduce the

chance of any probe carry-over. Humidity was held constant at 45%. Printed slides were baked for one hour at 130˚C (22 in. Hg) and stored at room temperature away from light.

2.3. Target preparation

Amplification of the 16s rDNA gene was accomplished by pairing one of eight sense primers with the EubA1518R primer to produce products of varying lengths (Fig. 1). Primers 16s517Rev and 16s336Fwd were used to amplify a separate 181 bp product. Each 25 µl PCR reaction contained 1X Buffer (Fisher Scientific), 1.5 mM $MgCl_2$, 200 µM dNTP, 1 Unit *Taq* polymerase, 400 nM of each antisense and sense primer, and 30 ng of *E. coli* genomic DNA or 5 µl boiled lysate. Cycling conditions for PCR reactions included initial denaturation for 5 min at 95˚C combined with 30-35 cycles of denaturaton (95˚ C for 30 sec), annealing (62˚ C for 45-60 sec), and extension (72˚C for 60 sec) followed by a final extension of 72˚C for 10 min. Agarose gel electrophoresis was used to confirm amplicon length. The PCR amplicon generated from 16s_fwd001 and EubA1518R primers (1,517 bp) was nick translated as follows. The initial PCR product was ethanol precipitated, resuspended in deionized water, and nick translated using a commercial kit (BioNick, Invitrogen). The reaction was allowed to proceed 2 h at 17˚C and labeled products were ethanol precipitated before being resuspended in 1X hybridization buffer (5X Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrrolidine, 0.1% bovine serum albumin], 4X SSC [60 mM NaCl, 0.6 mM Na-citrate, pH 7.0]).

2.4. Hybridization and detection.

Slides are initially blocked with TNB 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) at 23˚C for 30 min prior to target hybridization. PCR amplicons were diluted by combining 5 µl PCR product, 17.5 µl of 2X hybridization buffer (10X Denhardt's solution, 8X SSC), and 12.5 µl deionized water per well. This target solution was then heated to 95˚C for 2 min to denature double stranded DNA and was stored on ice until applied to the slide (35 µl well^{-1}). Upon target application, slides were placed in a humidified chamber (50 ml conical tube with a filter paper moistened in nanopure water) and incubated in a water bath for at least 4 hours at 55˚C. After incubation, targets were aspirated off and slides were rinsed in TNT (0.1 M Tris-HCl, 1.5 M NaCl, 0.05% Tween 20™, deionized water). All subsequent washes were in TNT buffer. A 1:100 dilution of streptavidin horseradish-peroxidase in TNB buffer was applied to the slides for 30 minutes (TSA™ kit). After additional rinses in TNT, slides were soaked in a 10% fetal equine serum (FES; Sigma Chemical Company; St. Louis, MO) solution to coat the slide surface for tyramide deposition. Biotinyl tyramide (TSA™ kit) was diluted 1:50 in 1X amplification diluent (TSA™ kit), and applied to the slides for 10 min. After rinsing, a dilution of 1:500 streptavidin Alexa Fluor® 546 conjugate (Molecular Probes, Eugene, OR) in 1X SSC and 5X Denhardt's was applied to the slides for one hour under reduced lighting. Slides were given a final rinse and dried in a Microarray High-Speed Centrifuge (ArrayIt, Sunnyvale, Ca). Hybridized targets were detected using an *ArrayWorx*^{e}™ scanner (Applied Precision, Issaquah, WA). *SoftWoRx*™ software (Applied Precision) was used to evaluate and quantify spot intensities and median values were averaged for each probe replicate. Each experiment was conducted 2-3 times and averages and standard deviations were calculated for replicate microarrays. Statistical tests were conducting using NCSS 2001 (Number Cruncher Statistical Systems, Kaysville, UT).

3. Results

We confirmed that coupling the EubA1518R primer with the eight different sense primers generated products of predicted size (Fig. 2A). We nick translated the longest product (1,517 bp) and hybridized the labeled targets to the array. All probes were visible thus demonstrating that all of the microarray probes were functional for subsequent experiments (Fig. 2B). Five-prime, terminally biotinylated amplicons of varying lengths (162-1,517 bp) were hybridized to microarrays under identical conditions. Signal intensity for each probe was measured for these hybridizations and compared with expected probe hybridization patterns (Fig. 3). All eight amplicons showed varying degrees of “missed” hybridization consistent with portions of the products not being available for interaction with their respective complementary probe sequences. This pattern of failed hybridization occurred with all of the products regardless of amplicon length. Interestingly, probes located in the 5′ region of the gene sequence (sense orientation) were most likely to fail regardless of the length of the PCR amplicon. Hence, amplicon length was a poor predictor of hybridization success.

Two explanations for probe failure included amplicon secondary structure interfering with hybridization and distance from the biotin moiety. For example, the fact that the biotin moiety was located on the 3′ terminus of the gene sequence whereas most probe hybridization failures involved the 5′ region of the sequence suggests a possible interaction between hybridization success and distance from biotin label. We already demonstrated that hybridizing nick translated products worked for all probes and this is consistent with relaxation of the secondary structure due to the nick translation process (Fig. 2B). To evaluate the relative significance of the location of the biotin moiety, we generated the longest PCR product (1,517 bp) using either a biotinylated sense or antisense primer. This produced amplicons having the

biotin moiety located at either the sense or antisense terminus of the gene sequence. In theory, having a biotinylated sense strand should not produce signal because the sense strand should not hybridize to the sense probes. Nevertheless, we have shown previously that this strategy works (Call, unpub. data), probably due to interaction of the labeled sense strand or labeled sense primer with the antisense strand that hybridizes to the probe. Amplicons biotinylated via the sense primer hybridized with no apparent loss of sensitivity relative to the antisense labeled amplicons and with little improvement in ability to hybridize to probes located in the 5′ region of the gene sequence (Fig. 4). Hence, distance from biotin moiety did not impact hybridization failure. The nick translation process produced many fragments of random length and thus this procedure should eliminate most amplicon secondary structure. Consequently, we concluded that unanticipated secondary structure rather than position of the biotin moiety was responsible for failure of targets to hybridize to their corresponding probe sequences.

While both shorter and longer amplicons potentially have secondary structure that adversely impact probe sensitivity, we surmised that shorter products would still be preferable when developing an assay. That is, assuming that a PCR reaction produces roughly the equivalent mass of product regardless of product length, then the molar concentration of a short product (e.g., 100 bp) could be as high as 10-fold greater than the molar concentration of a longer product (e.g., 1,000 bp). This assumes that molar concentration is the limiting factor in the sensitivity of the microarray detector. Even if molar concentration is a limiting factor, it is possible that this difference can be compensated to some extent by incorporating multiple labels into the target using nick translation. To test this idea we used primers 16s_517Rev and 16s_336Fwd from another study (Call et al. unpub. data) to generate a 181 bp amplicon that brackets the 16s_hyb489 probe on the array. We also prepared nick translated target from the

1,517 bp amplicon and both targets were serially diluted and hybridized to the array. The signal intensity was roughly parallel across the dilutions with no significant difference between the two labeling schemes (Fig. 5; $P = 0.68$; two-factor analysis of variance)

4. Discussion

When designing PCR assays that are coupled with microarray detectors, we have frequently observed probe hybridization failures. In this paper we demonstrate that the most consistent explanation for these failures is likely to be target secondary structure. Importantly, secondary structure appears to affect both long and short PCR amplicons so we were unable to circumvent this problem by altering amplicon length. Consequently, depending on the labeling system that is employed, the time needed to develop PCR-microarray assays could be unnecessarily extended because probe failures would require an iterative design process to identify sequences that are free from structural interference. Even when suitable probes can be identified, secondary structure could compromise detector performance. For example, there is considerable variation in the 16S rDNA gene between microbial taxa. It is possible that this variation could produce unanticipated secondary structure for some taxa (or strains), and consequently result in false negatives due to failed hybridizations.

We used signal amplification to enhance our ability to detect hybridized targets and we anticipate that secondary structure will also be a significant issue for non-amplified detection systems. While nick translation is one means to disrupt secondary structure, alternative labeling methods may work, but all of these methods can add significant cost to the assay relative to conjugated primers. For example, it is possible to directly incorporate labeled nucleotides into the amplicon during PCR amplification and this might provide sufficient steric interference to

prevent formation of secondary structure. Alternatively, directly labeled products could be fragmented by sonication to eliminate any secondary structure. Direct incorporation has the added advantage of reducing the time required to complete the detection steps, but conjugated nucleotides can add considerable expense to the assay depending on the molar concentration used in the PCR reaction. In addition, direct incorporation of fluorescent markers may compromise detector sensitivity relative to a signal amplification system. Random hexamer labeling would most likely alleviate secondary structure (Wang et al., 2002), although this method may not be as desirable for very short targets because of fewer potential binding sites for the short primers. Nick translation is not expected to work as efficiently with short amplicons either, but in our hands we have found no labeling difficulties based on target length. While amplicon length is not a good predictor of hybridization success, assays designed to amplify shorter products have the advantage of being more suitable when working with degraded template DNA.

Our data indicate that nick translation of a long PCR product does not compromise detector sensitivity (Fig. 5), and with a larger sample size it is possible that there is a statistical gain using nick translation (the power of our test was only $\beta = 0.15$). We did not attempt to make a definitive assessment of sensitivity differences between nick translation and our primer labeling systems. This was in part because the microarray detector is not the most limiting component of this type of assay system. A microarray detector can help to some extent as reported by Call et al. (2001a) where a microarray detector was shown to be 30-fold more sensitive than agarose gel electrophoresis for detecting a short PCR product. This detection advantage was based on a biotin conjugated primer and with this labeling system the sensitivity advantage is unlikely to be realized with longer amplicons. This is because the sensitivity of

agarose gel electrophoresis should increase proportionally with product length as more reporter molecules (e.g., ethidium bromide) intercalate into the PCR product. Nevertheless, under idealized conditions (purified DNA in buffer), a conventional microarray is perfectly suitable for detecting down to one copy of a bacterial genome when the assay relies on PCR amplification (Call et al., 2001a). Thus, the largest gains in overall assay sensitivity will be achieved at the level of sample preparation rather than at the level of amplicon detection (Chandler et al., 2000; Chandler et al., 2001). This is because complex sample matrices (e.g., food or environmental samples) generally produce low template yields and co-precipitating inhibitors can significantly impact assay sensitivity before the microarray detector is even considered. Thus, significant gains in overall assay sensitivity and reliability are more likely to be found at these earlier steps in the detection process.

In summary, when designing a PCR-microarray assay, all limiting factors including sample preparation and PCR amplification need to be considered with respect to improving assay sensitivity. Furthermore, the method used to label the final targets for microarray hybridization can significantly impact successful hybridization. In this study we demonstrate that secondary structure is a significant concern in the design and application of DNA microarray detectors and this challenge appears to be independent of amplicon length. Although nick translation adds additional expense to an assay, this method resolves problems due to secondary structure without compromising detector sensitivity.

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Table 1. Primer and probe sequences used in this study.

Primer name	Sequence (5′ -- 3′)
16s_fwd001	AATTGAAGAGTTTGATCATGGCTCA
16s_fwd224	CAGATGTGCCCAGATGGGATTA
16s_fwd442	CGGGGAGGAAGGGAGTAAAGTT
16s_fwd618	TCAACCTGGGAACTGCATCTGA
16s_fwd798	GGTAGTCCACGCCGTAAACGAT
16s_fwd974	GAACCTTACCTGGTCTTGACATCC
16s_fwd1166	TAAACTGGAGGAAGGTGGGGAT
16s_fwd1356	TCAGAATGCCACGGTGAATACG
16s336Fwd	Biotin-AGACTCCTACGGGAGGCAGC
16s517Rvs	Biotin-ATTACCGCGGCTGCTGG
EubA1518R	Biotin-AAGGAGGTGATCCANCCRCA
Probe name	
16s_hyb083	TTGCTGTTTCGCTGACGAGTGG
16s_hyb275	GCGACGATCCCTAGCTGGTCTG
16s_hyb489	CGCAGAAGAAGCACCGGCTAAC
16s_hyb736	CCCTGGACGAAGACTGACGCTC
16s_hyb933	ACAAGCGGTGGAGCATGTGGTT
16s_hyb1136	GGCCGGGAACTCAAAGGAGACT
16s_hyb1205	GCCCTTACGACCAGGGCTACAC
16s_hyb1452	GGAGGGCGCTTACCACTTTGT

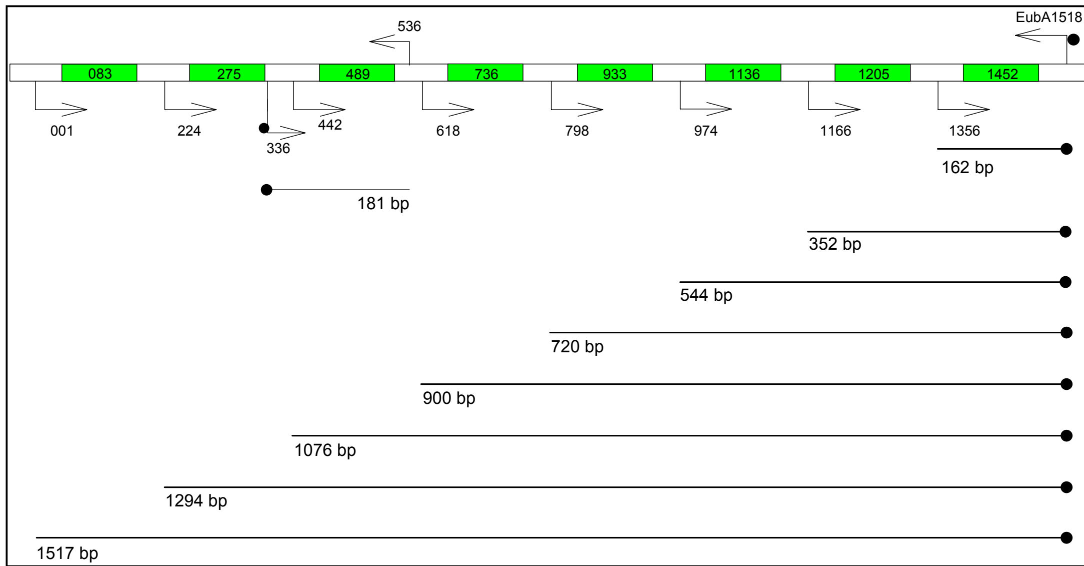
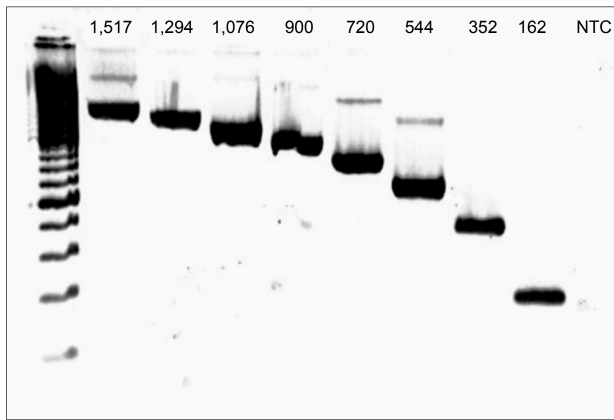


Figure 1. Primers (arrows) and probes (green) with corresponding amplicons (horizontal lines) used in this study (*E. coli*, Genbank AE000452). Biotin moieties are shown as closed circles.



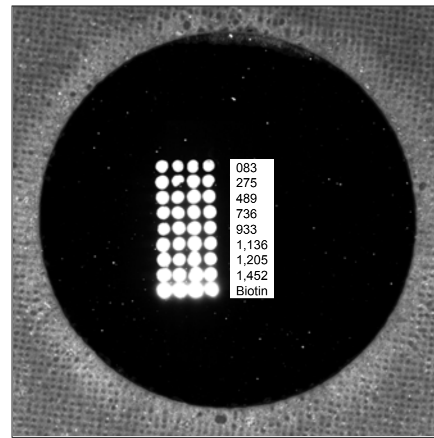


Figure 2. (A) Gel electrophoresis showing eight PCR amplicons with a 100 bp ladder. (B) An image of a nick translated 1,517 bp amplicon hybridized to the microarray showing the position of the eight probes and biotin control.

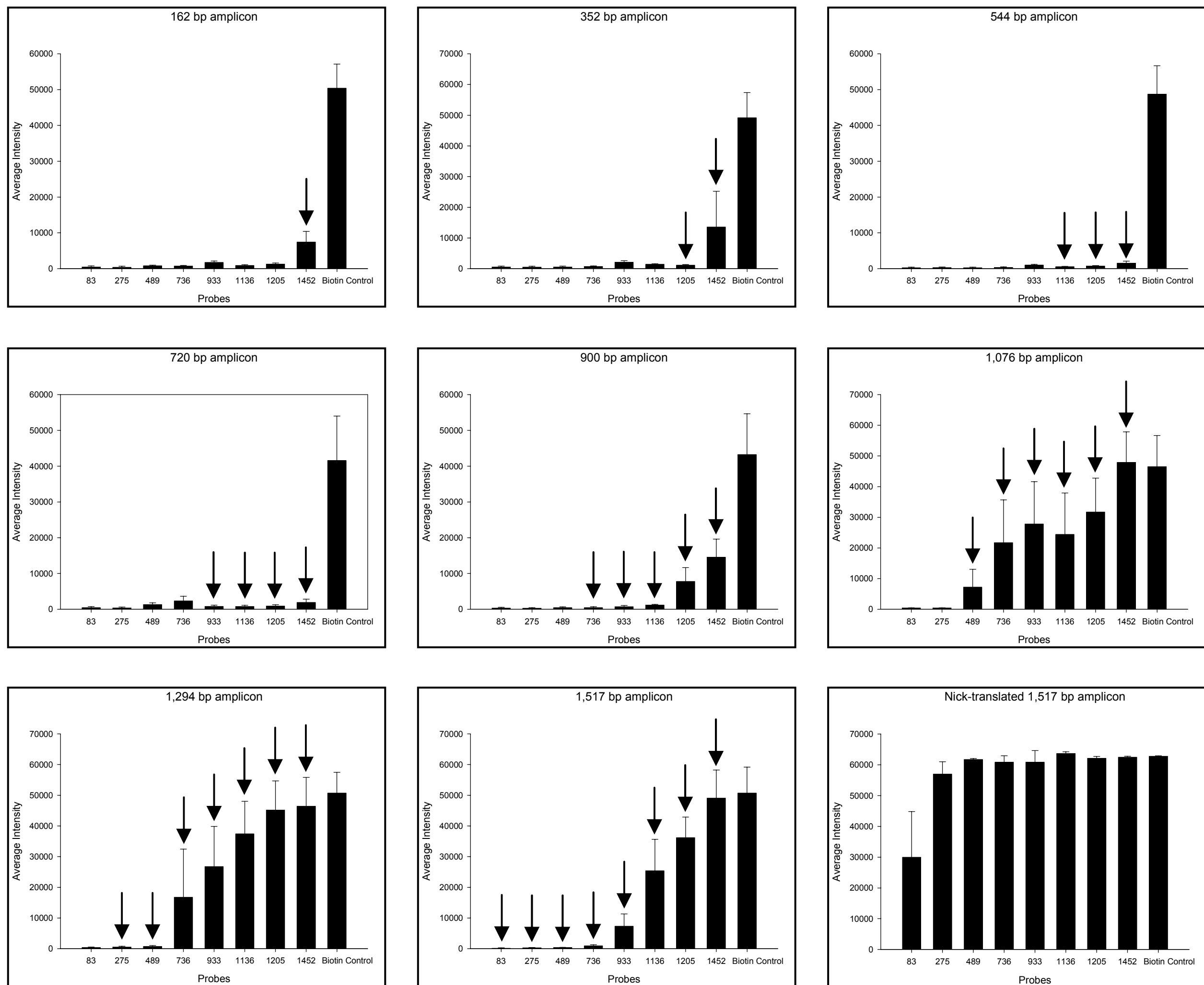


Figure 3. Probe signal intensity for eight different PCR amplicons. Expected amplicon hybridization is indicated with arrows. Bars represent the average signal intensity (± SEM) from three independent hybridization experiments.

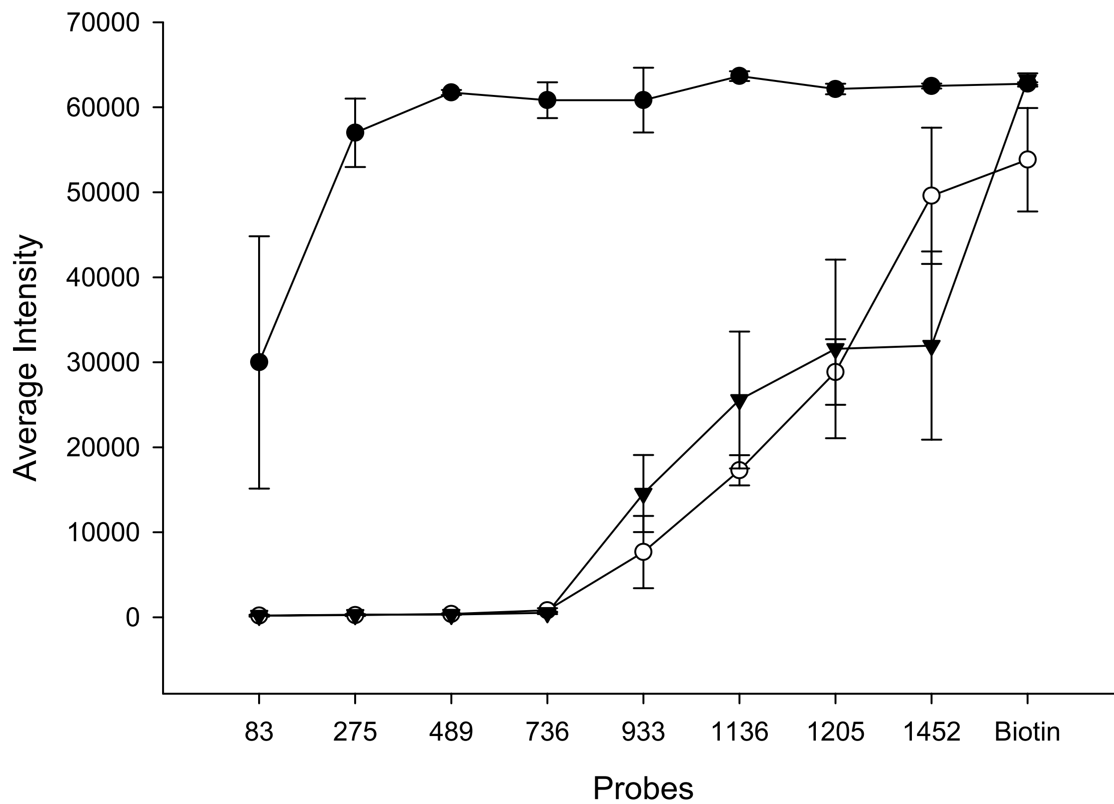


Figure 4. Probe signal intensity for 5′ (closed triangles) and 3′ (open circles) biotinylated 1,517bp amplicons as well as the 1,517 bp nick translated amplicon (closed circles). Each point represents average signal intensity (± SEM) from three independent hybridization experiments.

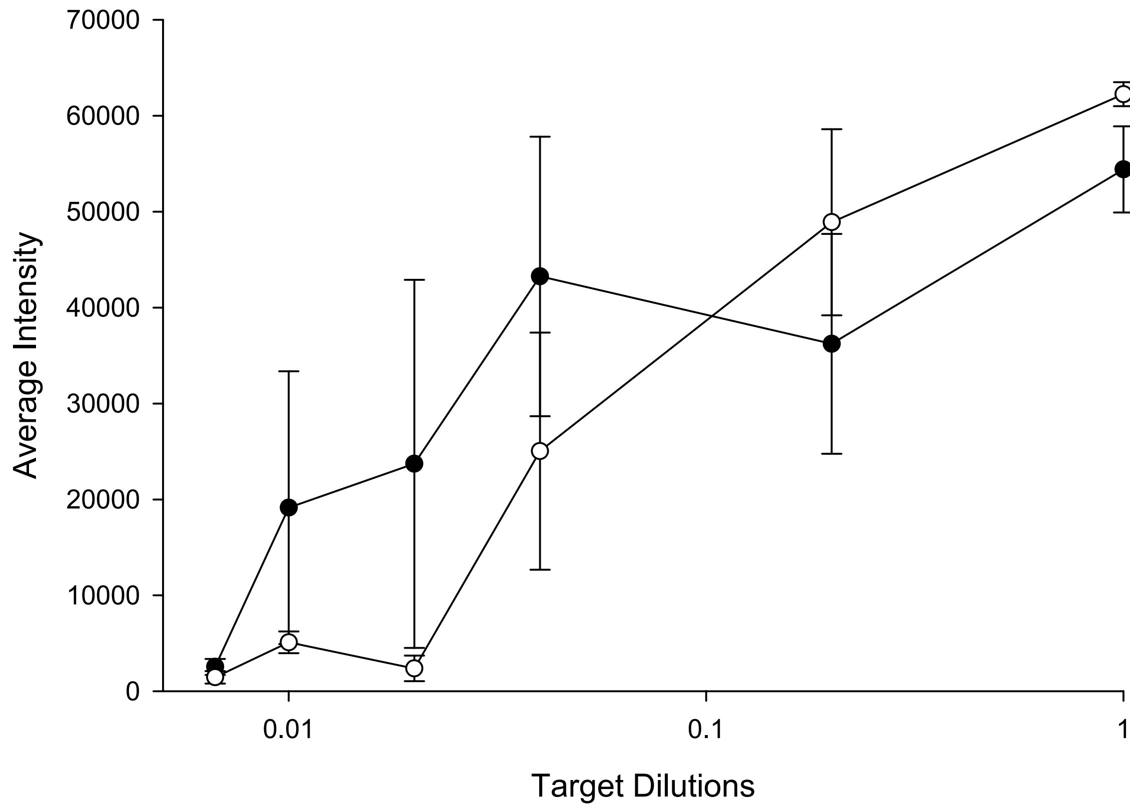


Figure 5. Probe signal intensity at different concentrations of a nick translated (closed circles) 1,517 bp amplicon and a terminally biotinylated 199 bp amplicon (open circles). Each point represents the average signal intensity (± SEM) from 2-3 independent hybridization experiments.