Comparison of different labeling methods for the production of labeled target DNA for microarray hybridization

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Abstract

Different labeling methods were studied to compare various approaches to the preparation of labeled target DNA for microarray experiments. The methods under investigation included a post-PCR labeling method using the Klenow fragment and a DecaLabel DNA labeling kit, the use of a Cy3-labeled forward primer in the PCR, generating either double-stranded or single-stranded PCR products, and the incorporation of Cy3-labeled dCTPs in the PCR. A microarray that had already been designed and used for the detection of microorganisms in compost was used in the study. PCR products from the organisms Burkholderia cepacia and Staphylococcus aureus were used in the comparison study, and the signals from the probes for these organisms analyzed. The highest signals were obtained when using the post-PCR labeling method, although with this method, more non-specific hybridizations were found. Single-stranded PCR products that had been labeled by the incorporation of a Cy3-labeled forward primer in the PCR were found to give the next highest signals upon hybridization for a majority of the tested probes, with less non-specific hybridizations were found. Hybridization with double-stranded PCR product labeled with a Cy3-labeled forward primer, or labeled by the incorporation of Cy3-labeled dCTPs resulted in acceptable signal to noise ratios for all probes except the UNIV 1389a and Burkholderia genus probes, both located toward the 3′ end of the 16S rRNA gene. The comparison of the different DNA labeling methods revealed that labeling via the Cy3-forward primer approach is the most appropriate of the studied methods for the preparation of labeled target DNA for our purposes.

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1. Introduction

The introduction of molecular methods in the 1980s based on the comparative analysis of 16S rRNA gene sequences has allowed environmental
microbiology to enter a new age (Olsen et al., 1986; Insam, 2001; Peplies et al., 2004). Nucleic acid microarrays represent one of the most recent advances in molecular technologies, allowing a high-throughput format for the parallel detection of 16S rRNA genes from environmental samples (Liu et al., 2001; Small et al., 2001; Bodrossy and Sessitsch, 2004). DNA chip technology offers the possibility to analyze an entire array of microorganisms, concerning their presence or absence in a particular environmental sample in a single experiment. The presence of both variable and conserved regions in the 16S rRNA gene has resulted in this molecule being used in the majority of microarray studies in microbial ecology (Liu et al., 2001). The conserved regions are nearly invariant among different bacteria, whereas the variable regions often provide discrimination between different species (Woese, 1987). As a result, oligonucleotide probes can be designed to hybridize to different groups or species of bacteria by targeting regions of greater or lower sequence conservation (Stahl et al., 1988; Amann et al., 1995).

The various processes of microarray experiments (fabrication, hybridization, and detection) can be conducted in different ways. Oligonucleotide and cDNA probes can be affixed on nylon membranes, glass slides, gel elements, or microbead surfaces (Guschin et al., 1997; Spiro et al., 2000; Small et al., 2001; Lievens et al., 2003). Recently, electronic microarray technology has also been described as a potential alternative in bacterial detection and identification (Barlaan et al., 2005). Targets for hybridization may be PCR products, oligonucleotides, genomic DNA, or cDNA, and thus, environmental microbial communities can be studied with or without PCR amplification (Call et al., 2003). Detection sensitivity is an important parameter in environmental microarray studies. In order to increase the sensitivity level of detection, many researchers include a PCR amplification step to obtain sufficient amounts of labeled target DNA (Loy et al., 2002). PCR, although increasing the sensitivity of the microarray, can also introduce potential biases to the analysis (Felske et al., 1998; Polz and Cavanaugh, 1998; Cook and Sayler, 2003). The labeling of DNA for microarrays can be done in different ways. Wang et al. (2002, 2004) and Tiquia et al. (2004) successfully used a random priming technique with the DNA polymerase Klenow fragment in a post-PCR process to label the DNA. This method is reportedly reliable and reproducible, although can result in an increased amount of non-specific hybridization (Bodrossy et al., 2003). We have previously reported on the successful design and development of a microarray for the investigation of compost microbial communities using a Cy3- or Cy5-labeled 8F primer in the PCR (Franke-Whittle et al., 2005), based on a similar approach to that reported in the study of Peplies et al. (2003). This method was found to label DNA relatively quickly, and inexpensively.

In this earlier study, we systematically optimized our oligonucleotide microarrays by comparing different microarray fabrication parameters, such as the effect of a T-spacer, different hybridization temperatures, the amount of oligonucleotide spotted on the slide, and the effects of different hybridization buffer concentrations (Franke-Whittle et al., 2005). In this study, the efficiency of different methods of labeling 16S rDNA for microarray hybridizations is compared.

2. Materials and methods

2.1. Source of bacterial strains and culturing conditions

The pure cultures included in the study were Burkholderia cepacia and Staphylococcus aureus. B. cepacia was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), and S. aureus from the Institute for Hygiene und Social Medicine, University of Innsbruck. Cultures were grown on nutrient agar plates, which contained (per litre deionised water) 3 g meat extract, 5 g peptone, and 15 g agar, pH 7.

2.2. Design and evaluation of oligonucleotide probes

The ARB software package (Ludwig et al., 2004) was used to design the genus and species level oligonucleotide probes included on the microarray. The
PROBE_FUNCTIONS tool and the PROBE_MATCH tool for specificity testing were used in the design process. All possible probe mismatches to non-target organisms were centralized in order to minimize the risk of unspecific binding. The GeneRunner program (http://www.generunner.com) was used to calculate GC%, melting temperature, and formation of secondary structures. Probe sequences were subjected to a BLAST analysis (Altschul et al., 1991), and further evaluation of the specified probe sequences was done by searching for matches between the probe and its potential targets within the rRNA sequences in the RDP (Ribosomal Database Project) II database. The sequence, specificity, and Escherichia coli position of the probes of interest in this study are shown in Table 1.

2.3. Microarray manufacture and processing

The microarray used in this study was based on the array designed and described previously (Franke-Whittle et al., 2005). Additional probes were, however, added to it. Briefly, oligonucleotide probes for microarray printing were synthesized with a 12mer-poly-T-spacer at the 5′ end, and printed by Lambda GmbH (Freistadt, Austria). The 5′-terminal nucleotide of each oligonucleotide was amino modified with C6 MMT to allow covalent coupling of oligonucleotide probes to the aldehyde coated CSS-100 glass microscope slides (Genetix GmbH, München, Germany). Lyophilized oligonucleotides were resuspended in water and diluted to a printing concentration of 23 μM with 3 × SSC buffer prior to spotting onto the activated slide surface using a Piezo-printer (GeSim Nano-Plotter NP 1.2) with a “NanoTip” micropipetting tip. A volume of 0.3–0.4 nl of each probe was spotted in triplicate on slides. Both quality and hybridization controls were printed on the array.

2.4. Labeling methods

2.4.1. Preparation of labeled target DNA using a post-PCR labeling process

PCR amplification of 16S rRNA genes was conducted using the universal bacterial primers 8F (AGAGTTTGATCMTGGA) and 1492R (TACCTTGTTACGACT), as described by Buchholz-Cleven et al. (1997). Amplifications were performed in 50 μl volumes in a ThermoHybaid PCR Express thermal-cycler, with each standard reaction mix containing a final concentration of 1 × reaction buffer [16 mM (NH4)2SO4, 67 mM Tris–HCl pH 8.8, 1.5 mM MgCl2, 0.01% Tween 20] (GeneCraft, Münster, Germany), 0.2 μM of each 8F and 1492R primer, 1 × enhancer (Peqlab, Germany), 10 mM TMAC (tetramethylammonium chloride), 1.25 U BioTherm™ DNA polymerase (GeneCraft, Münster, Germany), 200 μM each

<table>
<thead>
<tr>
<th>Target organism</th>
<th>Probe name</th>
<th>Full name</th>
<th>Probe sequence (5′-3′)</th>
<th>Specificity</th>
<th>E. coli position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eucarya</td>
<td>KO 32c</td>
<td>S-D-Univ-1389-a-A-18</td>
<td>ACGGCGCGGTGTGACAAG</td>
<td>Bacteria, not 'Epsilonproteobacteria'</td>
<td>1389</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>KO 218</td>
<td>S-S-Saur-0070-a-A-19</td>
<td>GAAGCAAGCTTCTCGTCCG</td>
<td>Target species, S. haemolyticus, uncultured bacteria</td>
<td>70</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>KO 442</td>
<td>S-S-Saur-0185-a-A-18</td>
<td>CACCTTTGAAACCATGCCG</td>
<td>Target species</td>
<td>185</td>
</tr>
<tr>
<td>Low G+C</td>
<td>KO 319</td>
<td>S-*.LowGC-0350-a-A-20</td>
<td>GAAGATTCCCTACTGCTGCC</td>
<td>Low G+C bacteria</td>
<td>350</td>
</tr>
<tr>
<td>Burkholderia cepacia</td>
<td>KO 369</td>
<td>S-S-Bcep-463-a-A-20</td>
<td>TCATCCCCCGACTGTATTAG</td>
<td>Target species, B. multivorans</td>
<td>463</td>
</tr>
<tr>
<td>Burkholderia</td>
<td>KO 234d</td>
<td>S-G-Burk-1239-a-A-18</td>
<td>ACCCTCTTGTTCCGACC</td>
<td>Genus specific</td>
<td>1239</td>
</tr>
</tbody>
</table>

a Name of 16S rRNA gene-targeted oligonucleotide probe based on the nomenclature of Alm et al. (1996).
b Probe reference from Amann et al. (1990).
c Probe reference from Zheng et al. (1996).
d Probe reference from Hogardt et al. (2000).
dNTP, and sterile water. Template DNA (1–3 µl) was applied directly to each PCR reaction mix.

Thermal cycling was performed according to Peplies et al. (2003). After an initial denaturation at 95 °C for 5 min, amplification reactions were subjected to 10 min at 80 °C, 1 min at 48 °C and 3 min at 72 °C. Thermal cycling then proceeded with 33 cycles of 95 °C for 1 min, 48 °C for 1 min, and 72 °C for 3 min. Temperature cycling was followed by a final extension at 72 °C for 10 min.

Amplification products from two PCR reactions were pooled, purified with the GenElute™ PCR Clean-Up Kit (Sigma, Missouri, USA), and the amount of labeled DNA quantitated using the PicoGreen® dsDNA quantitation kit (Molecular Probes Inc., Oregon, USA) and a fmax Fluorescence Microplate Reader (Molecular Devices, CA, USA), as described by the manufacturer.

The DecaLabel DNA labeling kit (MBI Fermentas, Vilnius, Lithuania) was used to label products. Dried DNA (1300 ng) was resuspended in ddH2O and labeled according to the protocol of Loy et al. (2002). Briefly, DNA was denatured at 95 °C, and placed on ice. A deoxynucleotide mix comprising dGTP, dATP, dTTP, and Cy5-dCTP and Klenow Fragment exo- were added, and reactions were incubated at 37 °C. To increase the efficiency of labeling, the labeling step was repeated. Finally, dNTPs were added to each reaction, and reactions were incubated at 37 °C. Labeled DNA was purified with the QIAquick Nucleotide Removal Kit (Qiagen, Hilden, Germany) following the protocol of the manufacturer, with the exception that DNA was eluted twice from each column with 25 µl ddH2O.

2.4.2. Preparation of labeled double-stranded target DNA using a Cy3-labeled forward primer

Labeled target DNA was prepared by PCR amplification of 16S rRNA genes using a fluorescently labeled primer. The universal bacterial primers 8F, to which a Cy3-label was attached at the 5’ end, and 1492R, to which a PO4-group was conjugated at the 5’ end (Buchholz-Cleven et al., 1997) were used to amplify the nearly complete 16S rRNA genes of the bacteria of interest. PCR amplifications were prepared and performed as described above, except that reactions included 0.8 µM of the Cy3-labeled 8F forward primer, and 0.2 µM of the modified 1492R reverse primer.

Amplification products from two PCR reactions were pooled and purified, and the amount of DNA quantitated, prior to hybridization.

2.4.3. Preparation of labeled single-stranded target DNA using a Cy3-labeled forward primer

Labeled PCR product was generated, purified and quantitated as described in Section 2.4.2. In order to produce single-stranded DNA for the microarray, 1000 ng of purified PCR product was digested using 30 U Lambda exonuclease (Epicentre, Madison, Wisconsin, USA) in 1 × Lambda exonuclease buffer, at 37 °C for 2 h.

2.4.4. Preparation of labeled target DNA using Cy3-dCTP

Labeled target DNA was prepared by PCR amplification of 16S rRNA genes using Cy3-dCTP substitutes in the reaction mixture. PCR amplifications were prepared and performed as described in Section 2.4.1, except that reactions included 200 µM each dATP, dTTP and dGTP, 6.4 µM Cy3-dCTP, and 40 µM dCTP. The unlabeled 8F and unmodified 1492R primers were used in amplification reactions.

2.5. Hybridization

Five hundred nanograms of labeled PCR product from each bacterium was vacuum dried and resuspended in 11 µl of hybridization buffer consisting of 5 × SSC, 1% blocking reagent (Roche, Mannheim, Germany), 0.02 % SDS, 0.1% n-laurylsarcosine, and 5% formamide (Loy et al., 2002). The DNA from S. aureus and from B. cepacia was pooled into the one tube, 0.5 µl of a 0.5 M Cy3-labeled control oligonucleotide was added, and the mixture denatured for 10 min at 95 °C, before being placed on ice. Eighteen microliters was transferred onto a pre-chilled microarray (on ice) and covered with a glass cover-slip to guarantee a uniform wetting of the array surface. Arrays were placed into small plastic containers with hybridization buffer saturated blotting paper for equilibration. Hybridization was conducted at 51 °C for 4.25 h in a hybridization oven (Hoefer HB 400, CA, USA). After hybridization, slides were washed immediately at room temperature in washing buffers.
of increasing stringency for 3 min, initially in buffer 1 (1 × SSC, 0.2% SDS), followed by buffer 2 (0.1 × SSC, 0.2% SDS) and buffer 3 (0.05 × SSC). Finally, arrays were briefly submerged into ddH₂O, air dried, and kept in the dark until scanning. Experiments were conducted in triplicate.

2.6. Scanning of arrays

Scanning of the microarray slides was done as soon as possible after hybridization with a GenePix™ 4000B array scanner (Axon Instruments Inc., Foster City, CA). Scan power was set to 100% and PMT gain to 510 for clear visualization at 532 nm. Images were analyzed using the GenePix Pro 5.0 software (Axon Instruments Inc., Foster City, CA) and saved as multilayer tiff images. Results were transformed to Microsoft Excel (version 2002) spreadsheets for data analysis. In order to calculate the signal to noise ratio (SNR) for spots, the background value was subtracted from the median signal at 532 nm and divided with the standard deviation of the background [SNR = (F532Median – B532)/B532 SD2]. Signals were assumed to be above the absolute threshold if a SNR ≥3 was obtained.

2.7. Statistical analysis

Replicate results were tested for statistically significant differences at P ≤ 0.05 using the Mann–Whitney U-test. Significant differences between methods for the individual probes are indicated by small letters in the graph. Statistics were done using the SPSS 11.5.1 program for Windows (SPSS Inc. USA).

3. Results and discussion

This study was conducted in order to compare four different methods that can be used for labeling DNA for microarray experiments. The highest signals upon hybridization of the array with the differently labeled DNAs were mostly generated from PCR products labeled with the post-PCR labeling process (Fig. 1). This method resulted in the highest signals for 6 of the 8 probes included in this study. For 2 of the 8 probes (KO 32 and KO 234), the signal obtained using this method was significantly higher than that obtained by all other methods (P ≤ 0.05). However, with this method, we also found the highest level of non-specific hybridization, and this can be clearly seen in Fig. 2. Fig. 2a shows a microarray hybridized with single-stranded DNA labeled in the PCR using the Cy3-8F primer, and Fig. 2b shows the same array hybridized with DNA labeled by the post-PCR labeling process. Many non-specific signals can be seen on the second array. The probes targeting Acinetobacter sp., Nitrosomonas nitrosa, Campylobacter jejuni, the combined probes for Comomonas sp. and Acidovorax sp., and Thermomonospora chromogena/T. bispora, and a probe targeting Ralstonia solanacearum/Pseudomonas syzygii/blood disease bacterium all have between 1 and 10 base pair differences with the target region of both S. aureus and B. cepacia. However, using the post-PCR labeling process, these probes hybridized with the target DNA resulting in above threshold SNR values. The S. aureus and B. cepacia DNA was found to hybridize well with the ammonium-oxidizers probe KO 295, irrespective of which of the 4 labeling methods was used. There was only a single, centrally located base-pair mismatch between this probe and B. cepacia, and as this probe has also been found to hybridize with other non-target DNAs, it may be excluded from future microarrays.

According to Bodrossy et al. (2003), labeling via fragmentation can increase the background of a microarray and lead to an increased amount of non-specific hybridizations, despite being a reportedly reliable and reproducible labeling method. A possible explanation for why this method of labeling was found to generate higher levels of non-specific hybridization is that the labeling process employs random decanucleotides to bind to the denatured target DNA and generates smaller fragments with incorporated labeled dCTP bases. According to Tiquia et al. (2004), there is a bias associated with Klenow fragment labeling. These authors suspect that the Klenow fragment binds preferentially to nucleotide sequences with higher GC content, so that higher signals may not necessarily be indicative of higher population numbers of certain targets (personal communication). Optimization of this method by increasing the stringency of hybridization could likely result in a loss of non-specific hybridizations, but would also result in reduced signal strengths.
The signal hybridization intensities between slides generated with the post-PCR labeling process were found to differ considerably, indicating that there were variations in the efficiency of the fluorescence labeling of the PCR amplificates (Fig. 1). This has also been reported by Loy et al. (2002). Of the different labeling methods, the variation in SNR values between replicates was highest with this method. Another important factor that needs to be taken into consideration with this method is the cost involved. This post-PCR labeling process uses a DecaLabel DNA labeling kit and 1.7 μl of the Cy3-dCTP's per labeling reaction. The relatively high cost of Cy3-labeled dCTPs results in this method being more expensive than the other methods compared in this study.

The next highest SNR values obtained upon hybridization were found using the single-stranded Cy3-8F labeled PCR products, although this was not the case for the B. cepacia KO 368 and KO 369 probes. For the S. aureus KO 218 probe it was found that the highest signal was generated using a Cy3-labeled primer in the PCR. Interestingly, this probe has a target site close to the 5′ end of the 16S rRNA gene (E. coli numbering 70–88). Although the results obtained for the S. aureus KO 442 probe were marginally higher for the post-PCR labeling process, the difference in the results between using the single-stranded Cy3-8F labeling method and post-PCR labeling was not statistically significant. This probe also has a target site towards the 5′ end of the 16S rRNA gene (E. coli numbering 185–202). It would thus
seem that the probe target position on the E. coli 16S rRNA gene is important, and thus that the secondary structure of the 16S rRNA gene, and/or steric hindrance effects affect hybridization. It has also been reported by Peplies et al. (2004) that the secondary structure of the target DNA and the relative position of the probe binding site on the target molecule are important and affect the hybridization efficiency of a probe. Despite not producing as high SNR values for most probes as the post-PCR labeling process, the use of a labeled primer in the PCR produced labeled product in a shorter period of time, was found to produce less non-specific hybridization signals, and is also far more cost-effective than the post-PCR labeling process. As with all the labeling methods, variation in signal intensities between replicate slides was still seen (Fig. 1).

There were considerable differences between the signals generated by single- and double-stranded Cy3-8F labeled PCR products. The single-stranded products were obtained by subjecting the double-stranded PCR products to a digestion step to remove the phosphate labeled strand, and to leave a labeled single strand of DNA for hybridization. This method was adapted from the method of Peplies et al. (2003). There appeared to be a strong influence of the 16S rRNA gene probe target position on the signal intensity of hybridization when using the double-stranded PCR product in hybridizations. We found that for the probes with target sites closer to the 5' end of the 16S rRNA gene (e.g. KO 27, KO 218, KO 442, KO 319), the difference in the signals obtained for single- and double-stranded DNA was not statistically significant (P>0.05; Fig. 1). However, for probes with target sites further away from the 5' end of the gene (KO 368, KO 369), or closer to the 3' end of the gene, the differences were significant. In fact, for the Universal 1389a KO 32 and Burkholderia KO 234 probes which have target sites at 1389 and 1239 (E. coli numbering, respectively), either no signal, or signals below the threshold level were obtained when the PCR products were not digested, or when double-stranded PCR products generated using Cy3-dCTPs in the PCR were hybridized. In contrast, SNR values of 589 and 82 were obtained for the KO 32 and KO 234 probes, respectively, when single-stranded DNA was hybridized. All PCR products were, however, subjected to a 10-min denaturation prior to hybridization in order
to separate the strands. One explanation is that partial renaturation of the two strands results in an inaccessible- ability of the 3’ end target site to the probe. Another possibility is that a partially double-stranded PCR product has a steric hindrance effect, preventing successful hybridization at the 3’ end. This was not seen when hybridizing the single-stranded DNA, and thus it would seem that the confirmation of single-stranded and denatured double-stranded PCR products is different. Also, upon hybridization of denatured double-stranded PCR products generated using the post-PCR labeling process, there was no problem with hybridization of 3’ end targeted probes. This can probably be attributed to the fact that the Klenow fragment labeling generates smaller products, whose hybridization may not be affected by steric hindrance.

Guo et al. (1994) reported that the hybridization of single-stranded oligonucleotide probes is more efficient with single than double-stranded target DNAs. This is because hybridization of the double-stranded DNA to the support-bound oligonucleotide array will necessarily suffer from competition of the complementary strand with the oligonucleotide probes. Because of this, Blaškoviè and Baráè (2005) attempted asymmetric PCR to produce single-stranded PCR products. However, asymmetric PCR difficulties encountered by these authors resulted in the use of a normal PCR to generate double-stranded DNA targets for use in their hybridizations. Intense signals were reportedly found by Blaškoviè and Baráè (2005) after hybridization of probes with the double-stranded targets. No comments regarding any problems with hybridization of certain probes were made by these authors. Possibly the use of a shorter PCR fragment (750 bp) in their study may have avoided the problems that we encountered in this study.

With the exception of the Universal 1389a KO 32 and Burkholderia KO 234 probes which have target sites at the 3’ end of the 16S rRNA gene, labeling by the incorporation of Cy3-dCTPs into the amplicon during PCR amplification was found to result in good SNR values. For three of the probes examined, the SNR values were almost as high, or as high as the values obtained using the single-stranded 8F-primer generated PCR products. The B. cepacia KO 368 and KO 369 probes gave higher SNR values using this labeling method than the other methods, with the exception of the post-PCR labeling process. Different ratios of labeled: unlabeled dCTPs in the PCR were tested and it was shown that using lower amounts of the Cy3-dCTPs in the PCR resulted in weaker SNR values, while using higher amounts of the Cy3-dCTPs resulted in higher SNR values (results not shown). We used a concentration of 6.4 μM Cy3-dCTPs in PCR, and this was within the recommendations of the Fluorescent DNA labeling by PCR application note of Amersham Biosciences. Although using a higher concentration of Cy3-dCTPs did result in higher signals, the costs were also greater. The signals obtained with the 6.4 μM Cy3-dCTPs concentration were sufficient to determine successful hybridization (SNR of 17 for KO 442, and SNR values of 71–703 for the other probes). Conjugated nucleotides, although relatively expensive, do however allow for a relatively fast labeling process, and may provide sufficient steric hindrance to prevent formation of secondary structures that may decrease the success of hybridization (Lane et al., 2004).

The interpretation of microarray data is sometimes made difficult by the inherent variability in array experiments (Cook and Sayler, 2003). Many authors have reported variation in signal intensity from probes from microarray to microarray, and from day to day (Small et al., 2001). Small et al. (2001) reported on variation in signal intensities of quality control probes from array to array, and attributed these differences to the inherent variability in the analytical processes of microarray fabrication, hybridization and detection. Variable levels of hybridization to the different probes from a single target DNA is an expected consequence of using a single hybridization condition to evaluate an array of different probes, each having its own different kinetics of association and dissociation (Guschin et al., 1997; Liu et al., 2001). This was found in our experiments, with certain probes giving much higher signals (e.g. B. cepacia KO 369) than other probes (e.g. S. aureus KO 218 and KO 442). Due to the secondary structure of the 16S rRNA gene, some regions of the gene are less accessible to probe binding (Peplies et al., 2003). Other possible explanations include different probe melting temperatures, varying dG values of secondary structure formations of probes, and different lengths of probes.

In conclusion, we have compared 4 different methods for producing labeled DNA target for microarray studies, and have shown that all methods exhibit some
variation in the signal intensity of different probes between replicate samples. The post-PCR labeling process employing the Klenow fragment was found to give the highest signals, but also the highest level of non-specific hybridization, and the highest variation between probe signals on replicate slides. The use of single-stranded PCR products generated using a Cy3-labeled primer, as described in our previous publication (Franke-Whittle et al., 2005) was found to give the next highest SNR values for most probes. With this method, there was no obvious bias toward probe target sites located at either end of the 16S rRNA gene, as was seen with the hybridization of double-stranded PCR products labeled with a Cy3-8F primer, or by the incorporation of Cy3-dCTPs. Hybridization with single-stranded PCR products generated using a Cy3-labeled primer was also cost and time effective, not involving the use of expensive Cy3-dCTPs.

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