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Notes & Tips

High-sensitivity, polymerase chain reaction-free detection of microorganisms and their functional genes using 70-mer oligonucleotide diagnostic microarray

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Traditional application of the DNA microarrays to study gene expression [1] is being expanded to accommodate the rising need for high throughput, parallel microbial diagnostics [2,3]. Accessing the microbial diversity in environmental and clinical samples presents a major challenge in terms of both specificity and sensitivity [4]. Specificity requirements for microbial diagnostic microarrays (MDMs)[1] applied in environmental microbiology are the parallel and reliable detection of many microorganisms at the species, genus, or even higher taxonomic levels, whereas for the clinical MDMs species, subspecies and strain levels are to be targeted [2,3]. The relevant measure for sensitivity in this case is defined as the lowest relative abundance of the target group detectable within the analyzed community [2]. The current detection limit of MDMs lies in the range of 1–5% [5–7]. The option for the parallel detection of multiple marker genes (e.g., genes responsible for microbial pathogenesis, antibiotic resistance, and functional genes involved in certain pathways) is an additional bonus for MDMs, broadening their application potential.

The latest developments in the MDMs utilize long oligonucleotides (50–70 mer) [5–10]. Because of the increased length, long oligonucleotides display higher target-binding capacity than short oligonucleotides (15–30 mer), therefore increasing the sensitivity at the target level (detection of a single probe within a total genome/transcriptome background) [2]. However, threshold for the differentiation is at 85–90% sequence similarity (depending on the hybridization conditions), resulting in a reduced specificity of the probes compared to short oligonucleotides (where single mismatch discrimination is usually possible) [4,6,7]. This disadvantage can be compensated by targeting multiple marker genes, specific to the microorganisms that are to be detected. In such cases it is thus the targeted gene primarily defining the specificity rather than the probe itself.

Universal amplification and labeling methods of total community DNA [6–10] yield target representing the entire gene pool of the community without reduction in its complexity. Universal amplification and labeling methods exhibit very low levels of bias and enable the parallel detection of multiple genes without the cumbersome establishment of a multiplex PCR.

In this paper we report on the combination of long (70-mer) oligonucleotide microarray and universal genomic DNA amplification and labeling techniques, using readily available, affordable reagents and kits, in order to improve the sensitivity of the microarray-based microbial diagnostics. The method was tested with artificial mixtures of purified gDNA. The application with environmental DNA, potentially slightly fragmented, is likely to require some further optimization.

Salmonella-specific 70-mer oligonucleotide probes targeting the *invA* (invasion protein) and *sopB* (outer protein B) genes and a positive control 70-mer oligonucleotide probe targeting 16S rRNA gene of the *Enterobacteriaceae* family were designed (Table 1). The probes were custom-synthesized with and without a 5′ amino-C_6 linker (VBC-Genomics, Vienna, Austria). A 384-well flat-bottom plate was prepared with 30 μl of 50 μM oligonucleotide solutions in ArrayIt spotting buffer (TeleChem, Sunnyvale, CA). Samples were spotted with an OmniGrid spotter (1 Tele-Chem SMP3 pin) at 55% relative humidity (using the humidity controller of the spotter) and 21 °C, onto Corning

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[1] *Abbreviations used:* MDMs, microbial diagnostic microarrays; PCR, polymerase chain reaction.

Table 1
70-mer oligoprobes used in this study

Name	Sequence (5′–3′)
invA_1	aacagtgctcgtttacgacctgaattactgattctggtactaatggtgatgatcatttctatgttcgtca
invA_5	gaacgtgtttccgtgcgtaatatgaagttaattatggaagcgctcgcattgtgggcgccaagagaaaag
sopB_1	caaatgcgcgcatggcaatcgttgacccagccgggaaaaatgattcatttaaaaatccgcaataaagatg
sopB_2	agggaaaagggcgtatgcagttgggataccaagaatattcatcacgccaataatttgtggatgtccacggt
Ent_16S	tgccagcagccgcggtaatacggagggtgcaagcgttaatcggaattactgggcgtaaagcgcacgcagg

GAPS-II slides. Printed slides were stored unprocessed at room temperature in the dark until use.

Total genomic DNA (gDNA) was isolated from 10^8–10^9 *Escherichia coli* DH5α and *Salmonella enterica* serovar Senftenberg cultures that were homogenized using 0.1-mm glass beads (BioSpec Products, Bartlesville, OK) and bead mill homogenizer (Retsch, Haan, Germany) followed by organic extraction with phenol/chloroform as described previously [11]. For amplification and/or labeling experiments mixtures of *S. enterica* Senftenberg and *E. coli* gDNAs (ratios 1:1, 1:10, and 1:100) and pure *E. coli* gDNA control were used.

Twenty nanograms of total genomic DNA was amplified using isothermal Φ29 DNA polymerase (GenomiPhi DNA Amplification Kit; Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. gDNA templates (*S. enterica* Senftenberg and *E. coli* gDNA mixtures and pure *E. coli* gDNA control) were diluted to 20 ng/μl. One microliter of the template DNA was mixed with 9 μl of sample buffer (containing also random hexamers) and heat-denatured for 3 min at 95 °C. After denaturation samples were cooled to 4 °C on ice and 9 μl of reaction buffer and 1 μl of enzyme mix were added. Samples were briefly centrifuged and incubated overnight at 30 °C. After amplification samples were heat-inactivated (10 min at 65 °C) and amplified DNA was purified using ProbeQuant G-50 columns (Amersham Biosciences). Amplification of 20 ng total gDNA reproducibly yielded 4–6 μg of amplified DNA.

Total genomic or Φ29 amplified DNA was labeled using the BioPrime Array CGH Genomic Labelling System (Invitrogen, Carlsbad, CA) and Cy5-dCTP (Amersham Biosciences) with minor modifications of manufacturer's instructions. DNA template (1.5–1.8 μg) was resuspended in sterile MilliQ water to a final volume of 23 μl. Samples were mixed with 20 μl of 2.5× random primers solution (750 ng/μl random octamers) and heat-denatured for 5 min at 95 °C. After denaturation samples were cooled for 5 min on ice and 5 μl of 10× dCTP nucleotide mix, 1 μl Cy5-dCTP, and 1 μl exo-Klenow fragment (40 U/μl) were added. Samples were briefly centrifuged and incubated at 37 °C for 4 h. After incubation 5 μl of stop buffer was added and labeled targets were purified using the purification module supplied with the labeling kit. Labeled targets were eluted in 50 μl sterile MilliQ water. Labeling of of 1.5–1.8 μg template DNA reproducibly yielded 4–5 μg labeled target with an incorporation rate of one Cy5-dCTP per 20–30 nucleotides. The applied labeling method is actually expected to result in an amplification of the template DNA.

Immediately prior to the hybridization printed slides were blocked by acylation with succinic anhydride [12]. Hybridization was carried out as described before [13]. Labeled targets (entire labeling reaction, about 5 μg Cy5 labeled DNA in 50 μl) were heat-denatured (3′ at 95 °C). Hybridization buffer (prewarmed to 65 °C) was added to a total volume of 210 μl, with final concentrations of 40% formamide, 6× SSC, 1× Denhardt's reagent (Sigma, St. Louis, MO), 0.1% SDS, and 100 μg/ml salmon sperm DNA. Hybridization was performed overnight at 42 °C. After hybridization, slides were washed in a 2× SSC, 0.1% SDS wash solution for 5 min at 55 °C, followed by two wash cycles for 5 min in 0.2× SSC, and a final wash for 5 min in 0.1× SSC, all at room temperature. Slides were dried with an oil-free air gun and scanned immediately.

Microarrays were scanned at three lines to average and at 10 μm resolution with a GenePix 4000A laser scanner (Axon Instruments, Foster City, CA). Maximal laser power and PMT gain were set in order to detect 1% signal (saturation of the control 16S signal was ignored). Scanned images were saved as multilayer tiff images and analyzed with the GenePix Pro 6.0 software (Axon Instruments).

Amplification of 20 ng total gDNA (pure *E. coli* gDNA or *E. coli*–*S. enterica* Senftenberg gDNA mixtures) with Φ29 DNA polymerase reproducibly yielded 4–6 μg of amplified DNA. This amount was sufficient for at least two labeling and subsequent hybridization reactions. Direct labeling of 1.5 – 1.8 μg total genomic or Φ29 amplified DNA with exo-Klenow fragment resulted in 4–5 μg labeled target with incorporation efficiency of Cy-nucleotides at 25–30 nt/dye. It must be noted that environmental DNA is less intact in comparison with the genomic DNA purified from pure cultures which was used in the present work. As the efficiency of the isothermal amplification with Φ29 depends on the intactness of the template DNA, further tests and optimization steps are likely to be needed for the application of this method for real environmental samples. The increased complexity of environmental microbial communities may also pose further challenges, again requiring further optimization. However, this is beyond the scope of the present paper.

Using the above-described universal amplification/labeling methods in combination with long oligonucleotides (70 mer), low-background Corning slides, and stringent hybridization and wash conditions we were able to obtain a clear signal corresponding to a 1% of target organism (*S.* Senfenberg) within a mixed (artificial) community. *Salmonella*-specific probes reproducibly exhibited signal

intensities three to five times stronger than that of the slide background. No difference in the hybridization efficiency between amino-labeled and unmodified 70-mer oligos was observed, indicating that the costly 5′ amino modification can be avoided under the conditions used. Supplementary fig. 1 displays an example hybridization. These results represent a considerable improvement over current detection limits that are around 5% of the total population analyzed [6,7]. Until now 1% detection limit was achieved only with the help of tyramide signal amplification labeling [5], which is a technically more demanding and more expensive approach. Tyramide signal amplification was, furthermore, shown in the cited study [5] to improve the detection limit from 5 to 1%, raising the possibility that the combination of our approach with tyramide signal amplification may result in a further improvement in sensitivity.

Previous studies [6] indicated that the combination of universal labeling and long oligonucleotides could improve the sensitivity at the community level. It was shown that the detection limit of microarrays was related to the DNA input into the labeling reaction. The detection limit of randomly labeled DNA from the pure cultures is estimated to be approximately 5–10 ng, whereas the estimated detection limit in the presence of community nontarget DNAs lies between 50 and 100 ng [6,7]. However, employing universal amplification methods, we were able to improve the overall sensitivity at the community level by detecting 1% of the community DNA corresponding to approximately 18 ng of target DNA when using only exo-Klenow-labeling protocol, and 100 pg by combining Φ29 amplification and exo-Klenow labeling.

The above-described method, combining long oligonucleotides and universal amplification/labeling methods, makes it possible to detect target sequences representing 1% of the whole microbial community. The method relies on commercially available, relatively moderately priced reagents. Furthermore, the method allows for a minimal input (i.e., 20 ng of total DNA suffice for two microarray hybridization) which is of great advantage in both environmental and clinical microbiology where samples may often be limited, yielding low amounts of DNA. Universal amplification with random primers allows for the parallel detection of multiple genes without introduction of the PCR bias, and without a need for the development and optimization of multiplex PCR. High sensitivity, low input DNA requirement, minimal bias, and the potential to screen for hundreds of target sequences in parallel make this approach well suited for the application with microbial diagnostic microarrays.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2005.08.014.

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