detection of small numbers of nucleic acids would be possible when amplification and detection are carried out simultaneously on the cartridge (9). Detection methods such as nanoparticles (3) might be another way to improve the sensitivity and eliminate the need for amplification. Our principle for analysis of the detection limit can easily be adopted for any other microarray system and could be a first step for the identification of suitable technologies for potential applications in routine screening of samples without amplification.

REFERENCES

1. **Blohm, D.H. and A. Guiseppi-Elie.** 2001. New developments in microarray technology. Curr. Opin. Biotechnol. *12*:41-47.
2. **Edman, C.F., D.E. Raymond, D.J. Wu, E. Tu, R.G. Sosnowski, W.F. Butler, M. Nerenberg, and M.J.Heller.** 1997. Electric field directed nucleic acid hybridization on microchips. Nucleic. Acids Res. *25*:4907-4914.
3. **Härmä, H., T. Soukka, and T. Lövgren.** 2001. Europium nanoparticles and time-resolved fluorescence for ultrasensitive detection of prostate-specific antigen. Clin. Chem. *47*:561-568.
4. **Heller, M.J., A.H. Forster, and E. Tu.** 2000. Active microeletronic chip devices which utilize controlled electrophoretic fields for multiplex DNA hybridization and other genomic applications. Electrophoresis *21*:157-164.
5. **Petrik, J.** 2001. Microarray technology: the future of blood testing? Vox. Sang. *80*:1-11.
6. **Shi, M.M.** 2001. Enabling large-scale pharmacogenetic studies by high-throughput mutation detection and genotyping technologies. Clin. Chem. *47*:164-172.
7. **Small, J., D.R. Call, F.J. Brockman, T.M. Straub, and D.P. Chandler.** 2001. Direct detection of 16S rRNA in soil extracts by using oligonucleotide microarrays. Appl. Environ. Microbiol. *67*:4708-4716.
8. **Taniguchi, M., K. Miura, H. Iwao, and S. Yamanaka.** 2001. Quantitative assessment of DNA microarrays—comparison with Northern blot analyses. Genomics *71*:34-39.
9. **Westin, L., C. Miller, D. Vollmer, D. Canter, R. Radtkey, M. Nerenberg, and J.P. O'Connell.** 2001. Antimicrobial resistance and bacterial identification utilizing a microelectronic chip array. J. Clin. Microbiol. *39*:1097-1104.
10. **Zammatteo, N., L. Jeanmart, S. Hamels, S. Courtois, P. Louette, L. Hevesi, and J. Remacle.** 2000. Comparison between different strategies of covalent attachment of DNA to glass surfaces to build DNA microarrays. Anal. Biochem. *280*:143-150.

The authors wish to thank Elise Langdon-Neuner for assistance in editing the manuscript. *Address correspondence to Dr. Klaus Zimmermann, Baxter BioScience, Industriestrasse 72, A-1221 Vienna, Austria. e-mail: zimmerk@baxter.com*

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Thomas Eiter, Klaus Zimmermann, and Friedrich Scheiflinger
Baxter BioScience
Vienna, Austria

Internal Controls for Normalizing DNA Arrays

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General challenges of DNA array hybridization experiments are the specificity and reproducibility of these assays. We have previously addressed specificity through a combination of enzymatic labeling of DNA probes with the possibility of detecting several targets simultaneously by array hybridization (3–5). High signal-to-noise ratios, caused by the specificity in the enzymatic labeling reaction, were obtained (4). However, reproducibility is based on the signal intensities of the hybridization regions alone. Thus, experiment-to-experiment variation is dependent on the spot shape/size, amount of probe used, and the hybridization conditions. If the assay is not highly standardized, then a relatively high variance could be introduced into the results.

Here 5′ Cy5-labeled internal control probes were included in the assay. The control probes were identical to the probes used in sequence-specific labeling. The signals from the sequence-specific labeled probes (TAMRA-ddCTP; Perkin Elmer Life Sciences, Boston, MA, USA) are measured relative to the internal control probes (Figure 1). The

relative signals obtained are hybridization independent. Factors influencing the hybridization will affect equally the internal control probe and corresponding sequence-specific labeled probe.

The 5′ Cy5-labeled internal control probe system was tested in a 16S rRNA array hybridization experiment. *Lactococcus lactis* subsp. *lactis* INF (Department of Food Science Culture Collection, Agricultural University of Norway) L2, *Propionibacteria jensenii* INF P303, and *P. freudenreichi* ISU (Department of Food Science and Human Nutrition, Iowa State University) P59 were used in the assay. Approximately 10^8 cfu of cells were disrupted mechanically with glass beads (106 µm; Sigma, Steinheim, Germany) in a Fast-Prep bead beater (Bio 101; Stratagene, La Jolla, CA, USA). The treatment was performed twice for 20 s at maximum speed with a 5-min pause at 4°C between each treatment. The DNA was subsequently purified with the DNeasy® tissue kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations, and the DNA was eluted in a 100-µL volume (Treimo et. al., manuscript in preparation).

A PCR-amplified 18S rRNA fragment from *Saccharomyces cerevisiae* was used as a competitor for rigorous quantification. The competitive fragment was generated with the primers KRU1 yeast (5′-TGGCTCAGATTGAACGCTGGCGGCGCCCATTCGGGTCTTGTAATTG-3′) and Amp 1520r yeast (5′-CCGRTACGGYTACCTTGTTACGACTTGCCCCCTTCTCTAAGCAGATCC-3′). These primers contain yeast 18S rRNA-specific 3′-ends and 5′-ends identical to the primers KR 1 and AMP 1520R, described below. The amplification conditions used to generate the competitor were 95°C for 10 min, five cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 90 s, and then 40 cycles of 95°C for 30 s and 72°C for 2 min. A 7-min extension step of 72°C was included. The reaction mixture contained the same components as described below.

A dilution series ranging from 10^5 to 10 copies of the competitive fragment was added to 1-µL aliquots purified DNA. The DNA was subsequently PCR-amplified by using the two primers KR 1 (5′-TGGCTCAGATTGAACGCTGGCGGC-3′) and AMP 1520R (5′-CCGRTACGGYTACCTTGTTACGACTT-3′), which are located in regions of the 16S rRNA that are conserved among *Lactococcus* spp. and *Propionibacteria* spp. (Treimo et al. manuscript in preparation). The reaction mixture contained 10 pmol each of the PCR primers and 25 µL 2× SYBR® Geen PCR Master Mixture (Applied Biosystems, Foster City, CA, USA), and analytical-grade water (Sigma) to a final volume of 50 µL. The reaction parameters used were 95°C for 10 min, then five cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 90 s, and then 40 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 90 s. A 10-min extension step of 72°C was included.

Twenty microliters of the reaction were dephosphorylated with the addition of 4 U shrimp alkaline phosphatase (USB, Cleveland, OH, USA) and incubation at 37°C for 30 min. The enzymatic activity was inactivated by treatment at 95°C for 10 min. These products were then used for the cyclic labeling reaction. The specific probes used in this reaction are described in Table 1. The cyclic labeling conditions were as follows: 1× Thermo Sequenase™ reaction buffer (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), 10 pmol each specific probe, 100 pmol ddNTP (except ddCTP) (Roche Molecular Biochemicals, Manheim, Germany), 100 pmol TAMRA-ddCTP, 16 U Thermo Sequenase DNA polymerase, and 22 µL phosphatase-treated PCR product in a final volume of 60 µL. The labeling included 10–25 cycles of 95°C for 15 s, 50°C for 15 s, and 60°C for 1 min. Finally, 1 pmol 5′ Cy5-labeled probes, the same as the probes used in the cyclic labeling, was added to each sample.

SigmaScreen™-coated (Sigma) or CMT-GAPS™-coated (Corning, Corning, NY, USA) slides for microarrays

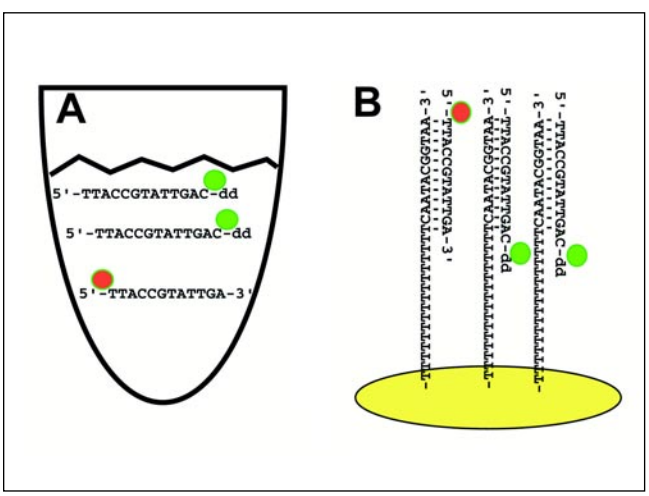

Figure 1. Schematic representation of the relative signal measurements. A ratio between the sequence-specific labeled probes (green dot) and the internal control probe (red dot) is given in the liquid phase before hybridization (A). This ratio is also conserved after hybridization to the complementary sequences, independent of the hybridization conditions (B).

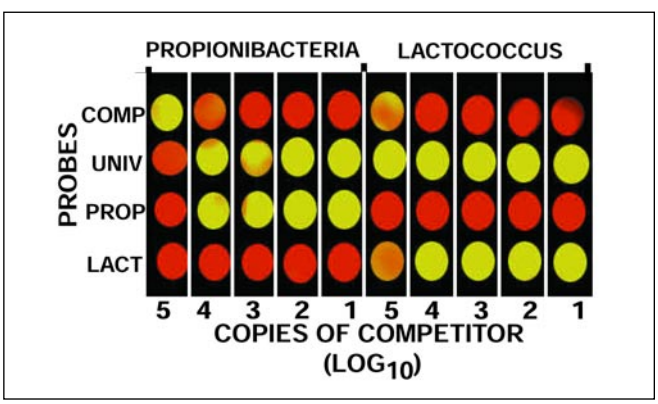

Figure 2. DNA array analyses. Each column represents a separate sample. Dilution series from 10^5 to 10 of the competitor were analyzed for constant amounts of DNA from approximately 10^6 cfu *P. freudenreichi* ISU P59 and *L. lactis* subsp. *lactis* INF L2, respectively. Five microliters of the labeling mixture were used for each hybridization experiment. The TAMRA signal (green) was detected with a 580-nm bandpass filter, using a green laser (533 nm) for the excitation. The Cy5 signal (red) was detected with a 670-nm bandpass filter, using a red laser (633 nm) for the excitation. The internal sections of the respective spots in the arrays are shown.

Table 1. Probes Applied in the Sequence-Specific Labeling

Name	Sequence (5′→3′)	Specificity: *Lactococcus* spp.	Specificity: *Propionibacteria* spp.	Yeast Competitor
COMP	CGCGGCTCCACTCC-TGGTGGTG	–	–	×
UNIV	GTCGGARTCGCTAGT-AATCGRGATCAG	×	×	–
PROP	GCGTGCGGGATGAC-GGCCTT	–	×	–
LACT	GAAGAACGTTGGTGA-GAGTGGAAAGCTCAT	×	–	–

were used for the DNA array hybridization experiments. One microliter (100 pmol/µL) of the following primers were spotted on the slide: 5′-T_{15}CACCACCAGGAGTGGAGCCTGCG-3′ (complemetary to COMP), 5′-T_{15}AAGGCCGTCATCCCGCACGC-3′ (complementary to PROP), 5′-T_{15}CTGATCYGCGATTACTAGCGAYTCCGAC-3′ (complementary to UNIV), and 5′-T_{15}ATGAGCTTTCCACTCTCACCAACGTTCTTC-3′ (complementary to LACT). The slides were then treated according to the respective manufacturer's instructions. The hybridizations were done overnight using 100 µL ArrayHyb™ LowTemp hybridization buffer (Sigma) and 1–10 µL labeling mixture. One slide was divided into 20 independent reaction chambers using a Cross Blot Dot Blot hybridization chamber (Sebia, Moulinaux, France). The washing was performed following the recommendations for the ArrayHyb LowTemp hybridization buffer. Fluorescence was then detected directly using a Typhoon™ scanner (model 8600; Amersham Biosciences). The resulting signals were analyzed with the ImageMaster Array Software.

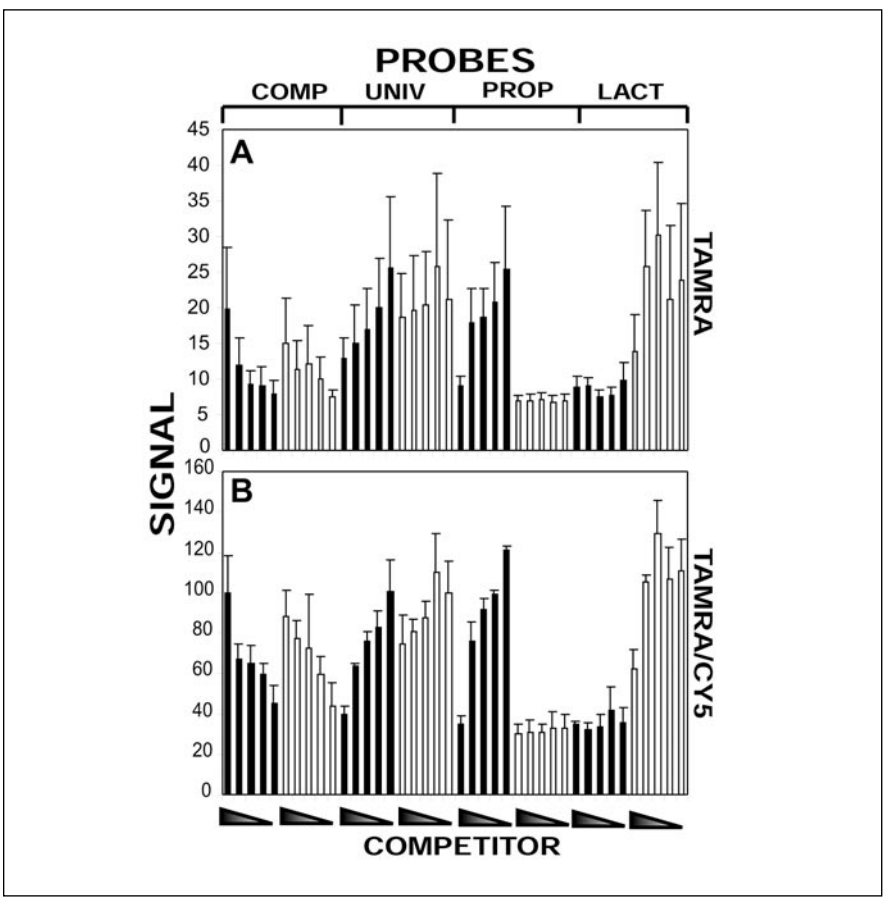

Figure 3. Uncorrected and normalized DNA array hybridization signals. The uncorrected signals (A) are volume values (1×10^5) for the TAMRA signal, as determined with the ImageMaster™ Array Software (Amersham Biosciences), while the corrected signals (B) are relative values for the TAMRA signals divided on the Cy5 signals. The black bars represent signals for *P. freudenreichi* ISU P59, and the white bars represent *L. lactis* subsp. *lactis* INF L2 signals. The dilution series for the competitor are 10-fold from 10^5 to 10 copies (represented by triangles) against constant amounts of bacterial DNA (corresponding to 10^6 cfu). Standard deviations are based on four replicates.

We questioned whether the inclusion of a Cy5 internal standard would reduce the sample-to-sample variation caused by the differences in hybridization conditions of the samples tested. The dilutions of the competitor relative to constant amounts of *P. freudenreichi* P59 and *L. lactis* subsp. *lactis* L2 DNA were analyzed in our model system (Figure 2). When analyzing the signal from the sequence-specific labeling (the TAMRA-ddCTP signal) independently from the internal control (the Cy5 signal), a relatively high variation in the assay was obtained (Figure 3A). This variation is probably due to a combined effect of slide-to-slide variation and differences in cross-linking the spotted probes to the slides. However, when analyzing the ratio between the sequence-specific labeled probe and the internal control, most of the variation in the experiment was removed. The resulting assay had relatively low standard deviations (Figure 3B).

To simplify the assay, we tested whether the sequence-specific labeling could be done directly on the 5′ Cy5-labeled probe. However, using this approach, we obtained irreproducible results (not shown). The reason for this is probably the interference between the two fluorochromes located on the same molecule. Phenomena such as quenching and fluorescence resonance energy transfer (FRET) must be taken into account (2).

The concept of defined internal controls could also be used in microarray

gene expression experiments. Currently, such measurements are taken relative to a control sample (1). However, the problem with this approach is that there are no controls for the probes/genes when they are not expressed in the control sample, which is usually the case. By using internal control samples (e.g., prepared from the DNA used for spotting), defined internal controls can be constructed for all the probes used.

REFERENCES

1. **Meldrum, D.** 2000. Automation for genomics, part two: sequencers, microarrays, and future trends. Genome Res. *10*:1288-1303.
2. **Orlando, C., P. Pinzani, and M. Pazzagli.** 1998. Developments in quantitative PCR. Clin. Chem. Lab. Med. *36*:255-269.
3. **Rudi, K., S.L. Flateland, J.F. Hanssen, G. Bengtsson, and H. Nissen.** 2002. Development and evaluation of a 16S rDNA array approach for describing complex microbial communities in ready-to-eat vegetable salads packed in modified atmosphere. Appl. Environ. Microbiol. *68*:1146-1156.
4. **Rudi, K., O.M. Skulberg, F. Larsen, and K.S. Jakobsen.** 1998. Quantification of toxic cyanobacteria in water by use of competitive PCR followed by sequence-specific labeling of oligonucleotide probes. Appl. Environ. Microbiol. *64*:2639-2643.
5. **Rudi, K., O.M. Skulberg, R. Skulberg, and K.S. Jakobsen.** 2000. Application of sequence-specific labeled 16S rRNA gene oligonucleotide probes for genetic profiling of cyanobacterial abundance and diversity by array hybridization. Appl. Environ. Microbiol. *66*:4004-4011.

Address correspondence to Dr. Knut Rudi, MATFORSK, Norwegian Food Research Institute, Osloveien 1, N-1430 Ås, Norway. e-mail: knut.rudi@matforsk.no

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K. Rudi, J. Treimo[1], B. Moen, I. Rud, and G. Vegarud[1]
Norwegian Food Research Institute
[1]Agricultural University of Norway
Ås, Norway

Multicolor Post-PCR Labeling of DNA Fragments with Fluorescent ddNTPs

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A capillary-based DNA sequencer with multicolor fluorescence detection has high resolution, a broad dynamic range of signal detection, and is capable of automated operation and direct computer-aided data acquisition/handling. Thus, the system is increasingly used for high-throughput fragment analyses other than sequencing, such as microsatellite genotyping and quantitative single-stranded conformational polymorphism (SSCP) analysis.

The DNA fragments to be analyzed by the system, usually PCR products, must be fluorescent. A commonly employed method for labeling the fragments is to amplify the target fragments using fluorescently labeled primers. However, the synthesis of such primers is at least several-fold more expensive than the synthesis of unmodified primers, and the reduction of this cost is a considerable benefit for projects that involve the examination of a large number of fragments.

PCR products can be labeled during amplification by including fluorescent nucleotides in the reaction mixture. Internally labeled fragments are unsuitable for analyses that require the high resolution of fragments because the positions and number of incorporated labeled nucleotides vary from molecule to molecule, and the fragments are chemically heterogeneous (2,4). The products can also be labeled by amplification using primers with 5′ tags, followed by the re-amplification of the products using common fluorescent primers that anneal to the tags. This method requires lengthy primers for each target sequence, and the fidelity of amplification is uncertain because of the increased number of primers in the amplification reaction and extended thermal cycles.

We previously developed a post-PCR fluorescence-labeling method in which the target sequences were amplified by PCR using unlabeled primers carrying either 5′ ATT or 5′ GTT. The 3′-end residues of the products were then exchanged with fluorescent dUTP or dCTP by the terminal exchange activity of the Klenow fragment of DNA polymerase I (2). The procedure is suitable for examining loci by SSCP analysis of SNPs (3,7,8) or for multiplexed microsatellite genotyping (5,6).

We attempted to expand the repertoire of fluorophores for labeling, but some [Alexa Fluor® and Oregon Green® (Molecular Probes, Eugene, OR, USA), Cy (Amersham Biosciences, Piscataway, NJ, USA), Cy3, Fluorescein Chlorotriazinyl, Lissamine, Napthofluorescein, and Texas Red® (Perkin Elmer Life Sciences, Boston, MA, USA)] were poor substrates for the labeling, as examined by the previously described method (unpublished data and Reference 2). This is presumably because the nucleotides modified with the fluorophores were inefficient polymerization substrates, or the ends with modified nucleotides were too sensitive to the 3′ exonuclease activity of the enzyme. We then used fluorescent ddNTPs, which behave differently from their deoxy counterparts, as substrates for various DNA polymerases (1). The Klenow fragment of DNA polymerase I had a very low labeling efficiency (approximately 2%) compared to fluorescent dNTP labeling using the same enzyme, which was expected because the fluorescent ddNTPs are known to be poor substrates for the enzyme.

Genetically modified T7 DNA polymerase or *Taq* DNA polymerase efficiently incorporate ddNTPs (9–11) and are commercially available as enzymes for sequencing by the dideoxy-terminator method. However, these enzymes alone are unsuitable for our end-labeling purposes because they possess weak or no 3′ exonucleolytic activity, which is essential for the terminal exchange reaction. Therefore, we used a cocktail of two enzymes, one for end-cleavage and the other for end-filling.

We tested the Klenow fragment of the DNA polymerase I or the T4 DNA polymerase (New England Biolabs, Beverly, MA, USA) as the enzymes for end-cleavage and either Thermo Sequenase™ (Amersham Biosciences) or Sequenase (version 2.0; USB, Cleveland, OH, USA) for end-filling. The fluorescent nucleotides *N,N′*-diethyl-2′,7′-