Detection of Food Poisoning Bacteria in Fresh Vegetables Using DNA Microarray

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A DNA microarray is a very useful tool for detecting multiple bacteria simultaneously, because it can be used to analyze the characteristics of various bacterial genes on one glass slide. This study was mainly performed to establish an assay protocol using a DNA microarray for detecting three food-borne bacteria (*Salmonella enterica* serovar Enteritidis, *Yersinia enterocolitica*, and *Bacillus cereus*) in fresh vegetables. To create the DNA microarray for detecting these three species of food-borne bacteria, four previously designed oligonucleotide probes corresponding to the 16S rRNA sequences of each food-borne bacterium were spotted onto a single glass slide. Bean sprouts and lettuce were used as representative fresh vegetables. Diluted cultures of the three bacteria were inoculated into the juices obtained from the fresh vegetables, and the inoculum was cultured with Soybean-Casein Digest broth to amplify the targeted bacterial 16S rRNA. RNAs extracted from the cultures were fluorescently labeled and hybridized to the DNA microarray. All three bacteria could be specifically detected in the fresh vegetable samples using this assay protocol. This DNA microarray provides a convenient approach for the simultaneous detection of food-borne bacteria in fresh vegetables in combination with conventional culture methods.

Key words —— DNA microarray, food poisoning bacteria, fresh vegetable, 16S rRNA

INTRODUCTION

The average prevalence of reported food-borne illness from 1981 to 1995 was 28.01 per 100000 population in Japan. Food-borne illness of bacterial origin in Japan constituted 72.8% of the total.[1] Sales of minimally processed and fresh-cut produce are increasing for a variety of reasons, including public awareness of the health benefits associated with the consumption of produce, the year-round availability of vegetables, and an increase in the variety of commodities offered.[2] It is estimated that more than 2000 t of fresh salad is sold in Japan annually.

Because minimally processed vegetables can be contaminated with pathogens and because vegetables and fresh-cut produce are typically consumed without heat treatment or sanitation processes, the need for intervention methods to maintain the safety of minimally processed vegetables is imperative.[2] Simplifying and speeding up techniques to examine the microbiological quality of fresh vegetables would improve food safety and reduce health costs.

Several food-borne pathogens have been reported to be carried in fresh vegetables: *Salmonella* spp. have been involved in food-borne illness outbreaks related to the consumption of contaminated tomatoes, cantaloupes, watermelon, mustard cress, and bean sprouts.[3] *Yersinia enterocolitica* (*Y. enterocolitica*) is capable of growing at the colder temperatures that are heavily relied upon by the food industry to maintain product quality post harvest.[4] An outbreak of gastrointestinal illness caused by the consumption of home-grown raw vegetable sprouts contaminated with *Bacillus cereus* (*B. cereus*) has also been reported.[5]

In recent years, DNA microarray technology has been widely used in research on bacterial gene expression and as a procedure for the identification of environmental bacteria[6–8] because this technology can be used to analyze the characteristics of various genes simultaneously. DNA microarray technologies are powerful tools that can be used for the detection of multiple genes or target sequences on a single glass slide. The DNA microarray is therefore a very

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Table 1. Oligonucleotide Probes Used in This Study

Probe	Probe sequence (5′ to 3′)	Length (nt)	Position[a)]	Target bacteria
AE001	TGCTGCCTCCCGTAGGAGTC	20	337–356	Almost all bacteria
SSP003-L	ATCTCTGGATTCTTCTGTGGATGTC	25	993–1017	*S.* Enteritidis, *S. typhimurium*
YE002	AATCACAAAGGTTATTAACCTTTATGCCTT	30	451–480	*Y. enterocolitica*
BC001	AACTTCATAAGAGCAAGCTCTTAATCCATT	30	69–101	*B. cereus* group

a) *E. coli* (GenBank Accession No. J01695) numbering.

useful device for the simultaneous detection of various bacteria in samples (based on their genes or gene sequences).

We previously reported that five originally designed probes were useful for screening for bacteria associated with food poisoning. Five phylogenetic probes leading to 16S rDNA phylogenetic sequence analysis for the screening of food-borne bacteria were designed and their specificities were confirmed using RNA extracted from pure cultures of 13 species of food-borne bacteria, including *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis), *Y. enterocolitica*, and *B. cereus*.[9)]

In this study, a DNA microarray that immobilized four previously designed probes (AE001, SSP003-L, YE002, and BC001) for detecting food-borne bacteria was created, and an assay protocol for the simultaneous detection of *S.* Enteritidis, *Y. enterocolitica*, and *B. cereus* in fresh vegetables (bean sprouts and lettuce) was established.

MATERIALS AND METHODS

Bacterial Strains, Culture Medium, and Growth Conditions —— *S.* Enteritidis, *Y. enterocolitica*, and *B. cereus* were used as representative food-borne bacterial strains in this study. To prepare pure bacterial cultures, cells of each standard food-borne bacterial strain were inoculated into 10 ml of Soybean-Casein Digest broth (SCDB; 17 g of peptone for casein, 3 g of peptone for soybean, 5 g of sodium chloride, 2.5 g of dipotassium hydrogen phosphate, and 2.5 g of glucose per liter of laboratory-quality water) and incubated on a shaker platform at 30°C overnight.

Preparation of Fresh Vegetables —— Bean sprouts and lettuce were purchased from local food stores. To obtain juice from each vegetable, 125 g of bean sprouts and 300 g of lettuce were used. Each vegetable was cut into small pieces in a nylon bag under sterile conditions, mixed well, and then squeezed by hand. The juices were collected in a sterile tube. Two hundred and eighty microliters or 80 μl of each juice were equivalent to 1 g of bean sprouts or lettuce, respectively. The total bacterial cell number in each juice was enumerated by direct counting using a frame-spotting method.[10)] *S.* Enteritidis, *Y. enterocolitica*, or *B. cereus* was inoculated into the mixtures of each juice (280 μl of bean sprout juice or 80 μl of lettuce juice) and SCDB 10 ml at a final concentration of 10^5 cells/ml. The samples were then incubated at 30°C for 6 hr with shaking at 100 revolutions/min. A portion of each sample (0.5 ml) was used for RNA extraction.

RNA Extraction —— Total RNA was purified from bacterial cultures with an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen Inc., Hilden, Germany). DNA was digested with the RNase-free DNase Set (Qiagen Inc.) during RNA purification. Extracted RNA was quantified based on UV absorbance. The presence of RNA was confirmed by gel electrophoresis in 1.2% agarose gel containing ethidium bromide (1 μg/ml) and TBE gel running buffer.

Oligonucleotide Probe Sequence and Attachment on a Slide Glass —— The four oligonucleotide probes used in this study are shown in Table 1. The specificity of these probes was confirmed previously.[9)] The probes were spotted onto a glass slide in duplicate by NGK Insulators, Ltd. (Nagoya, Japan). The average size of the spots was 200 μm.

Microarray Hybridization and Detection —— One microgram of each extracted RNA was labeled according to the manufacturer's instructions for the ULYSIS Alexa Fluor 546 Nucleic Acid Labeling Kit (Molecular Probes, Inc., Eugene, OR, U.S.A.). The hybridization solution was prepared as follows: 1 μg of Alexa Fluor 546-labeled RNA, 15 μl of 20 × SSPE (0.2 M phosphate [pH 7.4], 3 M sodium chloride, 20 mM EDTA) and 5 μl of 5% (w/v) sodium dodecyl sulfate (SDS) solution were mixed and adjusted to a total volume of 50 μl with RNase-free water. The mixture was then heated at 65°C for 10 min, and

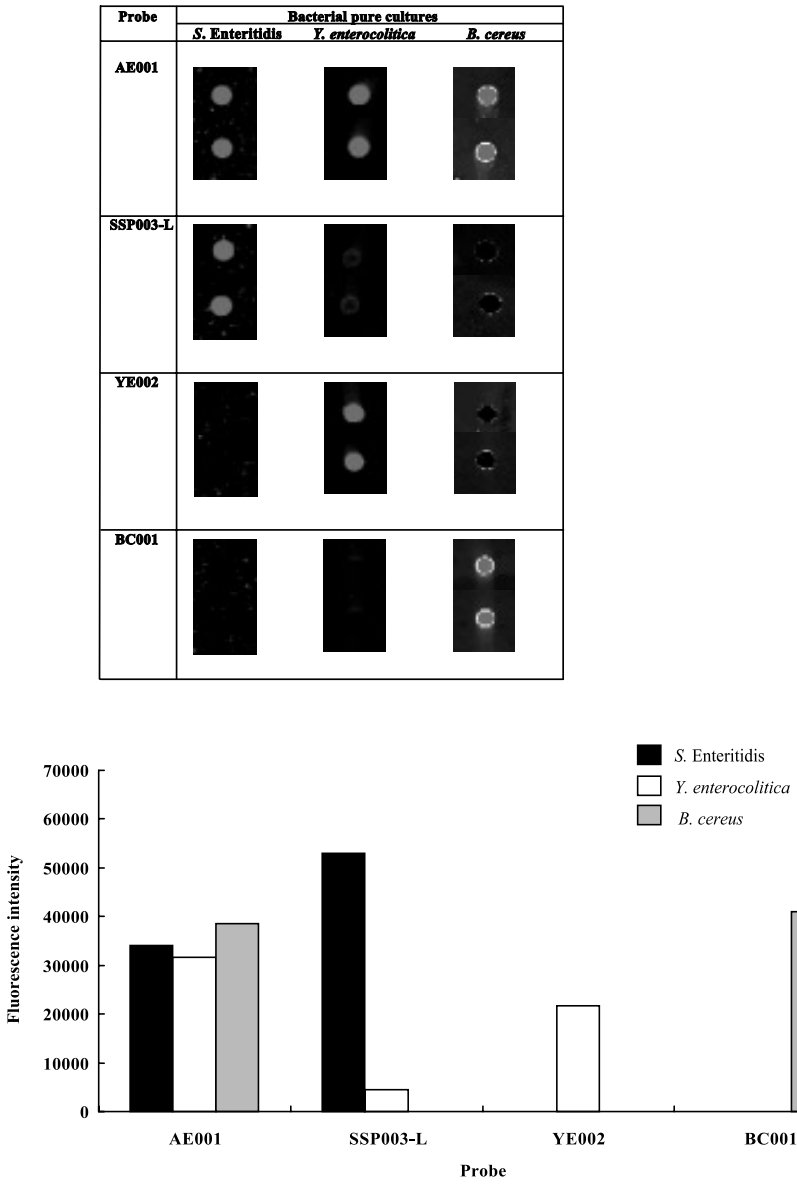


Fig. 1. Results of the Hybridization Assay of 16S rRNAs from Pure Cultures of Three Food-Borne Bacteria

Top panel: Hybridization images obtained with a GenePix 4000 laser scanner. Bottom panel: Quantitative fluorescence profile of the DNA microarray analyzed. The values are means of fluorescence values (n = 2).

40 μl of solution was poured onto the DNA microarray. The hybridization was performed at 45°C for 15 hr. The DNA microarray was washed five times with 2 × SSPE (20 mM phosphate [pH 7.4], 0.3 M sodium chloride, 2 mM EDTA) with distilled water and vacuum dried. After drying, the DNA microarray was scanned with a GenePix 4000 laser scanner (Axon, CA, U.S.A.). A laser light of wavelength 532 nm (PMT gain = 400 or 500) was used to excite the Alexa Fluor 546 dye. A fluorescent image was captured in a multi image tagged image-file format and analyzed with GenePix Pro 3.0 software (Axon).

RESULTS AND DISCUSSION

To confirm the reactivity of the four oligonucleotide probes for detecting the three food-borne bacteria, RNA extracted from pure cultures of the three bacteria was fluorescently labeled and hybridized to the probes immobilized on a glass slide (Fig. 1). All three bacterial 16S rRNAs hybridized to probe AE001 and generated a high fluorescence signal (> 30000). Therefore this probe was employed as a positive control probe for detecting bacteria. *S.* Enteritidis RNA, *Y. enterocolitica* RNA, and *B. cereus* RNA strongly hybridized to probe SSP003-L, probe

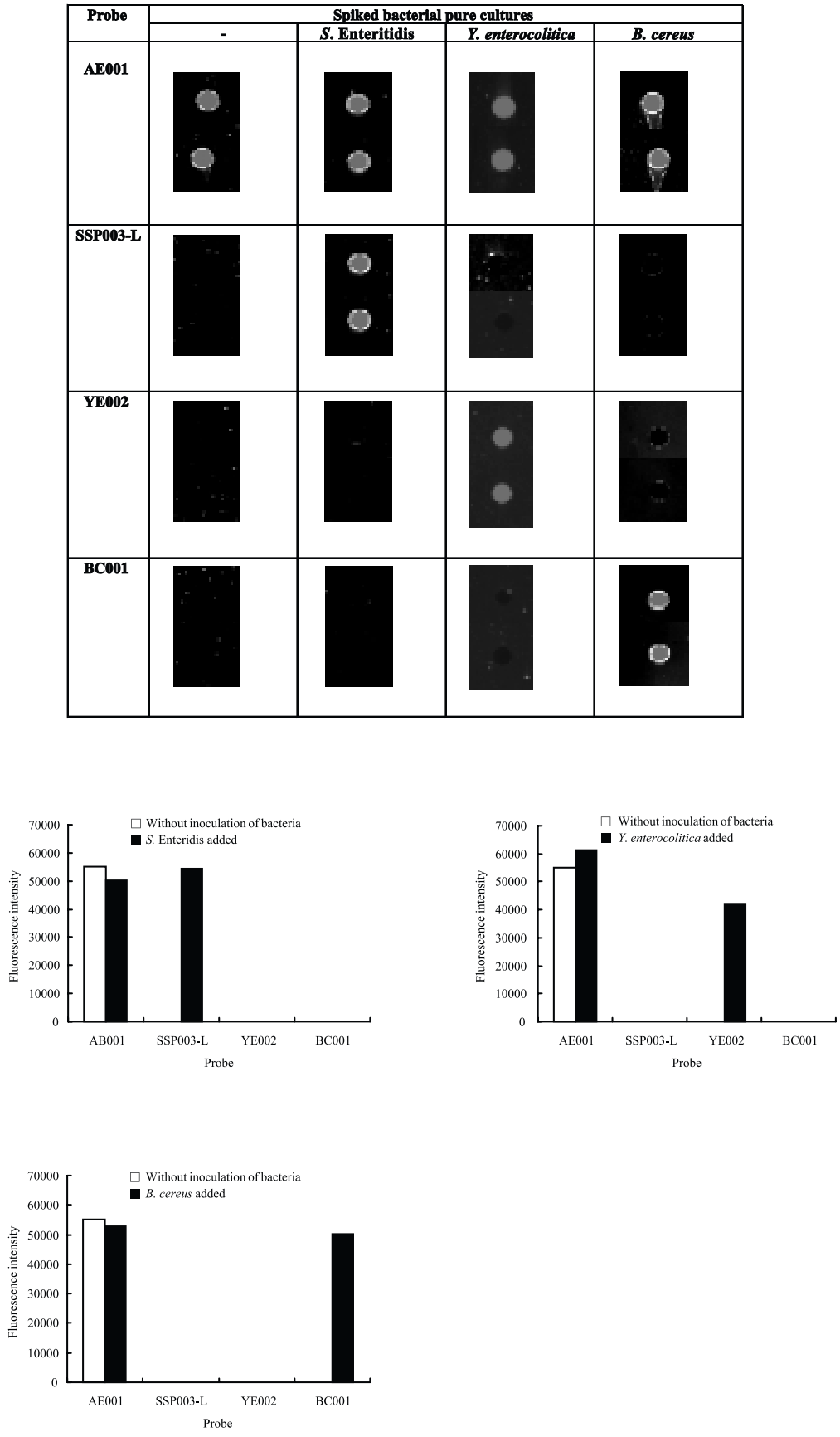
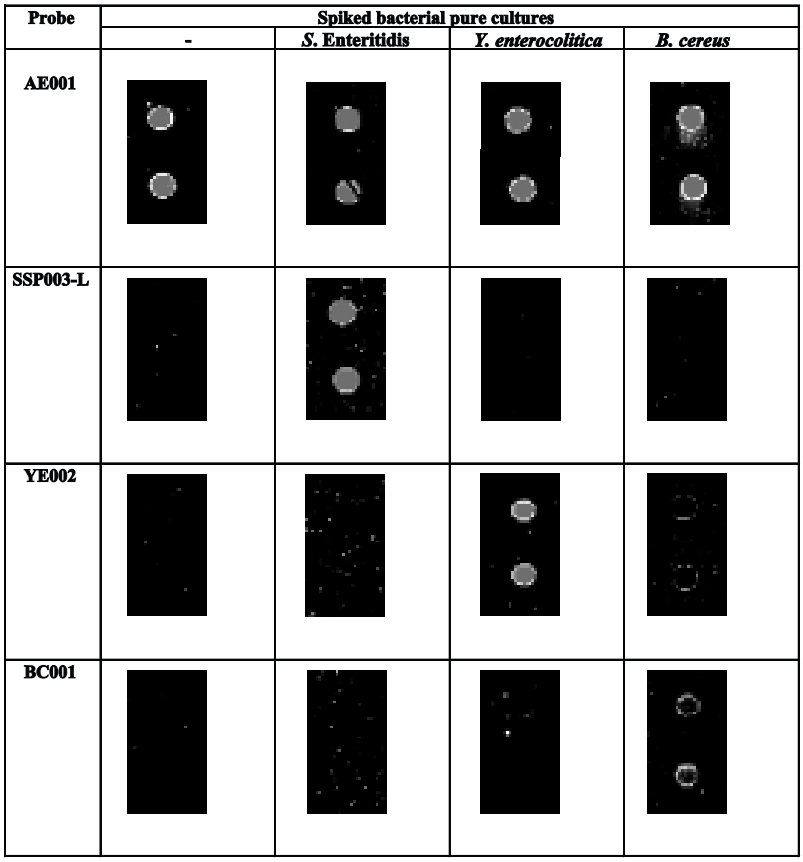
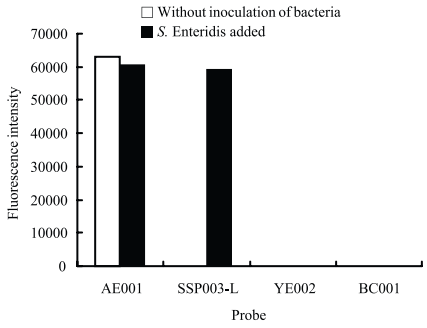
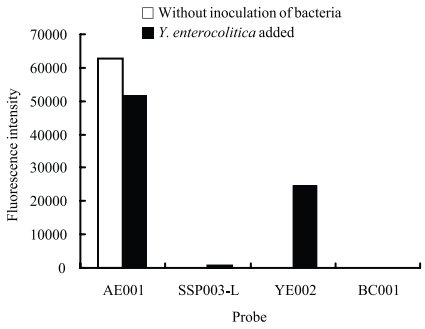


Fig. 2. Results of the Hybridization Assay of Three Food-Borne Bacterial 16S rRNAs from Model Bean Sprout Samples

Top panel: Hybridization images obtained with a GenePix 4000 laser scanner. Bottom panel: Quantitative fluorescence profile of the DNA microarray analyzed. The values are means of fluorescence values (n = 2).







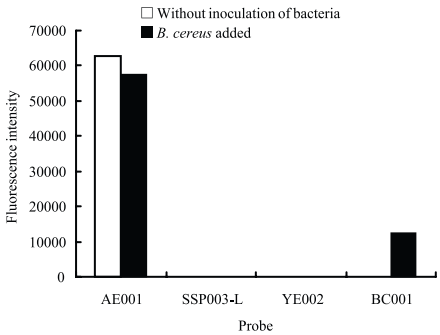


Fig. 3. Results of the Hybridization Assay of Three Food-Borne Bacterial 16S rRNAs from Model Lettuce Samples

Top panel: Hybridization images obtained with a GenePix 4000 laser scanner. Bottom panel: Quantitative fluorescence profile of the DNA microarray analyzed. The values are means of fluorescence values (n = 2).

YE002, and probe BC001, respectively.

Detection of 16S rRNA from the three species of food-borne bacteria from seeded bean sprout samples was examined. When RNA from the bean sprout sample that was not inoculated with bacterial culture was examined, only probe AE001 was hybridized. Probes SSP003-L, YE002, and BC001 generated high fluorescence values only when the existence of the targeted bacteria in the bean sprout samples was confirmed (Fig. 2). Seeded lettuce samples were also investigated. The hybridization results were similar to the results obtained from bean sprout samples (Fig. 3). The total bacterial cell number in 280 μl of bean sprout juice and 80 μl of lettuce juice was 4×10^6 and 4×10^5 cells, respectively. These cell numbers were nearly equal to those of bacterial cells inoculated into each sample.

For detecting bacteria using this DNA microarray, 1 μg of bacterial total RNA was used in this study. In this assay protocol, 16S rRNA was employed as surrogate marker for detecting food-borne bacteria, because up to 80% of bacterial RNA is rRNA, and one *Escherichia coli* (*E. coli*) cell can contain about 20000 copies of rRNA.[11] The generation time of representative pathogenic bacteria used in this study was as follows: *S.* Enteritidis, 20 min at 37°C;[12] *Y. enterocolitica*, 33–39 min at 32°C;[13] and *B. cereus*, 11–34 min at 42°C.[14] Although the RNA content varies greatly between bacterial strains and under different growth conditions, 1 μg of total RNA corresponds to approximately 1×10^7 bacterial cells in logarithmic phase. Therefore, if 10 cells/ml of the target bacterium are present in the sample, sufficient RNA required for this DNA microarray assay can be obtained from a sample incubated for approximately 10 hr.

The time required to perform the assay procedure for detecting food-borne bacteria using the DNA microarray was as follows: 1 hr to obtain RNA; 0.5 hr to label RNA with fluorescent dye; and approximately 17 additional hours to hybridize. The time required for detection, including the culturing of bacteria, is within 1 day, because the hybridization time of the DNA microarray can be reduced to approximately 6 hr (data not shown). The time required for bacteria detection using conventional culture methods is: *Salmonella* spp., 3 days; *Y. enterocolitica*, 5 days; and *B. cereus*, 1 day. Therefore this assay protocol using the DNA microarray can contribute to shortening the detection time of *S.* Enteritidis and *Y. enterocolitica*. This assay protocol might also be used for the simultaneous detection of *S.* Enteritidis, *Y. entercolitica*, and *B. cereus* in fresh vegetables.

This assay requires expensive RNA labeling reagents and sophisticated instrument (laser scanner for reading the assay results). These requirements may limit its broad application in ordinary laboratories. Hong *et al*. have developed a new oligonucleotide array assay method to eliminate the need for sophisticated equipment.[15] A digoxigenin-linked enzyme color development method was used in their study and the results could be evaluated with the naked eye. Applying such a method to our DNA microarray assay could make it easier to perform even in small laboratories.

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