Analysis of DNA Microarrays by Non-Destructive Fluorescent Staining Using SYBR® Green II

BioTechniques 29:78-81 (July 2000)

ABSTRACT

A simple, non-destructive procedure is described to determine the quality of DNA arrays before they are used. It consists of a preliminary staining step of the DNA microarray by using SYBR® green II, a fluorophore with specific affinity for ssDNA, followed by a laser scan analysis. The surface quality, integrity and homogeneity of each DNA spot of the array can thus be assessed. After this preliminary control, which may avoid further analytical steps that lead to the waste of precious biological samples, a fully reversible staining procedure is performed that produces an array ready for subsequent use.

INTRODUCTION

The use of microarrays is growing exponentially (5). The technology consists of dense arrays of DNA spots deposited on suitably prepared surfaces, mainly glass. Several formats have been proposed (10) that use oligonucleotides (3,6,8) or polynucleotides (11). DNA microarrays consisting of denatured DNA (usually longer than 200 bp) are being used for a number of analytical procedures such as differential expression (2,12) and mutation analysis. In essence, this recent analytical approach may be simply viewed as a miniaturization of the well-known dot blot (or reverse dot blot) analysis, having the merit to introduce a large parallelization (simultaneous monitoring) and automation of the analytical steps required for probing molecular diversity.

Membrane supports, which have been mainly used in conventional array formats (9), have now been largely superseded by modified glass surfaces, thus allowing a higher spot density (1). Several hundreds or thousands of single gene expression levels can thus be simultaneously monitored and generate a large set of data.

Complex chemical procedures required for the preparation and use of microarrays and the small amounts available of precious biological samples make it imperative that scientists develop and regularly apply suitable quality-control procedures to arrays before any hybridization. Eisen and Brown (4) recently described some of the main factors that affect microarray quality and proposed a series of control procedures to assess the overall performance of the glass array technique, as applied to the study of differential expression analysis. Accordingly, the uniformity of DNA deposition on the microarray can be determined by laser scanning after spotting, either to detect by light scattering the presence of salts (and thus of successfully spotted samples) on the surface or to detect heavily damaged, irregular glass surfaces.

This paper describes a new protocol to rapidly verify the existence, shape and relative fluorescence intensity of each spot in a DNA microarray after hybridization with SYBR® green II (Molecular Probes, Eugene, OR, USA), but before the hybridization with a biological sample. The first step is to use a fluorescent staining procedure using SYBR green II, an ultrasensitive fluorescent dye with a marked affinity for ssDNA (7). Laser scanning and fluorescence analysis of each spot in the array follow this process, and a destaining procedure follows this control analysis.

MATERIALS AND METHODS

Some of the following protocols have been adapted from those described by Eisen and Brown (4). For further details, see http://cmgm.stanford.edu/pbrown. All chemicals have been purchased from Sigma-Aldrich (Milan, Italy). PCR reagents are from Amersham Pharmacia Biotech (Milan, Italy) and Finnzyme (Oy, Finland).

Preparation of Target DNA

Target DNA was generated by PCR in a 50-µL reaction volume using a 96-well PCR Express™ thermocycler (Hybaid, Ashford, UK). Thermal-cycling parameters varied according to the tem-
plate and primers used. A portion of each PCR amplification product (5 µL) was examined by agarose gel electrophoresis, followed by ethidium bromide staining. Only PCR products showing a clear and strong band on UV transillumination were recovered by ethanol precipitation and resuspension in 15 µL 3× standard saline citrate (SSC) (450 mM NaCl, 45 mM sodium citrate, pH 7.0). The DNA concentration was determined using PicoGreen® reagent (Molecular Probes), a fluorescent nucleic acid stain useful for quantitating dsDNA in solution. The final concentration of DNA averaged 50 ng/µL. Samples were transferred into 96-well plates, which were sealed and stored at -20°C until used.

Preparation of Polylysine-Coated Glass Slides

Standard glass microscope slides (Sigma Aldrich) were pre-cleaned by immersion for at least 2 h in an alkaline wash solution consisting of 10% (w/v) NaOH and 57% (v/v) ethanol, followed by rinsing five times in double-distilled water. The slides were then gently shaken for 1 h in a coating solution consisting of 35 mM Poly-L-Lysine (Sigma Aldrich; 0.1% w/v in water), 35 mM filtered PBS and 280 mM double-distilled water. Coated slides were extensively washed with double-distilled water, centrifuged at low speed, (80× g) dried in a vacuum drying oven at 45°C for 10 min and then stored at room temperature in a tightly sealed slide box. Slides were used after at least two weeks to produce a sufficiently hydrophobic surface. This aging process is a key step in obtaining a suitable surface for array preparation.

Printing of DNA Microarrays

Target DNA samples in 3× SSC were spotted on the glass slides using a piezoelectric pipet (Nanoplotter System™, Gesim GmbH, Germany). The pipet was programmed to release about 10 nL DNA solution for each DNA spot. Spots were arrayed in a 20 × 20 arrangement (400 spots in a 1.5 × 1.5-cm square with a center-to-center spacing between spots of approximately 750 µm) or a 30 × 30 arrangement (900 spots in a 1.5 × 1.5-cm square with a center-to-center spacing of 500 µm). After deposition, arrayed DNA spots were completely dried by overnight incubation at room temperature in a covered box. Printed slides were rehydrated (DNA side down) in a plastic humid chamber (Sigma Aldrich) until spots glistened and then snap-dried at 100°C.

DNA was then cross-linked to the polylysine surface by UV irradiation using a Stratalinker™ oven (Stratagene, La Jolla, CA, USA) set at 65 mJ. Blocking of unreacted surface was obtained by incubating the slides in a solution of 1.5% sucemic anhydride, 90% 1-methyl-2-pyrolidinone and 43 mM sodium borate (pH 8.0). Surface-immobilized DNA was then denatured in boiling distilled water for 2 min, followed by immersion in 95% ethanol. The slide was then air-dried and stored until use in a closed, dust-free box at room temperature.

Quality Control of Nucleic Acid Deposition by SYBR Green II Staining

SYBR green II staining was used to visualize individual DNA spots on glass slides before hybridization. Staining was performed in a 50-mL Falcon® tube (Becton Dickinson, Franklin lakes, NJ, USA) by immersion of the slide for 2 min in a solution of SYBR green II diluted 1:10000 in TBE solution, pH 8.0. This diluted staining solution can be stored in a plastic tube and protected from light at 4°C for several weeks, following the manufacturer’s recommendations. The SYBR green II stock solution and the diluted SYBR green II reagent should be always handled with particular caution because this reagent is a potential mutagen. After staining, the slide was washed in TBE, double-distilled water and then air-dried. The fluorescent scanning device used for fluorescent analysis was a prototype instrument made by Quanta System (Milan, Italy) (De Bellis et al., manuscript in preparation) using a 488-nm excitation wavelength (argon ion laser). Any microarray scanner equipped with a 488-nm source is suitable to perform this analysis.

Data have been obtained and analyzed by image processing software developed in our laboratory. Briefly, the image from a scanner is filtered for small spurious isolated spots, and then an edge definition is performed to identify all relevant spots. A conditional dilation algorithm calculates background around every spot. The intensity for every detected spot is then calculated by integrating the signal within the spot.
and subtracting its own background. All data generated are saved in formatted tables that can be easily imported in commercial spreadsheet software. (De Bellis et al., manuscript in preparation). Any commercial software for microarray analysis can perform the same operations. Following image acquisition and processing, the fluorescent stain was completely removed from the slides by incubation at room temperature for 1 h in a solution of 1% SDS in TE, pH 7.5. After drying, the microarrays can be used for hybridization experiments.

Preparation of the Fluorescent Probe
A 795-bp probe was labeled by direct incorporation of a Cy3 dye during the PCR amplification procedure. Incorporation of the fluorescent nucleotide was accomplished using 200 µM dGTP, dCTP, dATP mixture, 40 µM unlabeled dTTP and 40 µM dUTP-Cy3 (Amersham Pharmacia Biotech). A small aliquot of labeled probe was examined by gel electrophoresis, and the remaining solution was purified by passage through a Sephacryl column (Amersham Pharmacia BioTech). The pellet, which was recovered after the precipitation step, was pink, indicating a successful labeling reaction. The pellet was stored at -20°C until used.

Hybridization and Detection of the Fluorescent Signal
The fluorescent DNA probe was dissolved in 15 µL hybridization solution consisting of 6x SSC, 2x Denhardt's solution (Sigma Aldrich), 50% formaldehyde and 0.4% SDS and then denatured at 90°C for 2 min. The array was covered with a glass slide, and the solution was introduced between the two surfaces to avoid the formation of bubbles. The slide was incubated overnight in a humidified chamber placed over an in situ hybridization block (PCR Express Thermocycler) maintained at 42°C. After hybridization, the slide was washed at room temperature by immersion and with gentle shaking in 1x SSC/0.3% SDS, 0.2x SSC and finally in 0.05x SSC. The slide was spin-dried and then analyzed by a laser scanning procedure using a 543-nm excitation wavelength (He-Ne laser) using the customized fluorescence scanner and image processing software.

RESULTS AND DISCUSSION
The staining protocol, which has been previously described above, was applied to the analysis of two DNA arrays. The same DNA target (i.e., a purified 765-bp PCR product with a concentration of 15 ng/µL) was spotted at different spot density on glass slides prepared according to the described procedure and then stained with SYBR green II. Figure 1 shows fluorescence signals obtained from an array whose spot density was 400 spots/2.25 cm² (20 x 20 spots on a 1.5 x 1.5-cm square). The analysis of fluorescence signals from each of the spots of the two arrays gave a CV of 10.5%. Figure 2 shows fluorescence signals from an array where different purified PCR products of variable length and concentration were spotted and then stained with the SYBR green II solution. Note that practically no fluorescence signals can be detected when the spotting solution was 3x SSC only (columns 3, 8 and 15). Clearly, this staining procedure seems suitable to assess the presence, shape and intensity of a single DNA spot of the array before any hybridization reaction is performed.

An important second issue for the subsequent use of the array is the possibility of achieving a complete destaining of the DNA spots. A 1% SDS/TE solution was found to be very effective for the complete removal of the fluorescent dye from the DNA spots. In fact, no difference was found in the level of background fluorescence between unstained and destained arrays of DNA spots. Our photon counting detector mounted on the laser scanning system detected 7.3 photons/pixel (CV 15.5%) on average on the slide in Figure 1 before SYBR green staining and 8.7 photons/pixel (CV 18.9%) after destaining, thus demonstrating the effectiveness of the destaining procedure. Figure 3 shows that the staining-destaining procedure has no detrimental effect on the hybridization of a Cy3-labeled DNA probe with a nucleotide sequence complementary to that of DNA spotted on the microarray of Figure 1. To further check the procedure, we analyzed microarrays by staining, destaining and restaining, gaining only a slight decrease of the signal (-12%) between the two staining steps. This confirms the non-destructive nature of this method.

SYBR green II is known to bind ss-DNA preferentially by an unknown mechanism (7), a particularly relevant feature since DNA targets are likely to be present in DNA spots in single-stranded form. In addition, when excited at 488 nm, this dye produces a strong emission signal centered at 520 nm. This avoids potential spectral interference with Cy3 (excitation 550 nm, emission 570 nm) or Cy5 cyanine dyes (excitation 650 nm, emission 670 nm) that presently are the most widely used fluorescence labels for differential expression analysis using microarrays. However, the destaining procedure is so efficient, as demonstrated by our results, that labeling with dyes with excitation at 488 nm can be considered, thereby expanding the fluorophores available for multicolor assays.

Other methods could be used for the quality control of microarrays. Microarrays obtained by amplification of clones with common primers can be easily checked by hybridization using one labeled primer, scanning and dehybridization. However, several microarrays have been obtained by primer-specific amplification and thus are not suited for this technique. On the other hand, the quality-control procedure we propose is suitable for any polynucleotide microarray, regardless of the source and sequence of the starting material.

REFERENCES

This work was supported in part by Italian CNR target project “Biotecnologie”, and MURST 40% “PRIN”. Address correspondence to Dr. Gianluca De Bellis, Consiglio Nazionale delle Ricerche, Istituto di Tecnologie Biomediche Avanzate, LIITA, via Fratelli Cervi 93, 20090 Segrate, Italy. e-mail: debellis@itba.mi.cnr.it

Received 20 December 1999; accepted 16 March 2000.

C. Battaglia, G. Salani, C. Consolandi, L. Rossi Bernardi and G. De Bellis
Consiglio Nazionale delle Ricerche
Istituto di Tecnologie Biomediche Avanzate
Segrate, Italy

Transmembrane Motility Assay of Transiently Transfected Cells by Fluorescent Cell Counting and Luciferase Measurement

ABSTRACT

Current in vitro assays used in assessing tumor motility could be improved by the development of a simple technique that would facilitate studies of the impact of specific genes on pharmacologically altered chemotaxis. We developed a technique that improves on the classic transwell assay by using fluorescence and luminescence to assess chemotaxis. In this transient transfection system, co-transfection of a reporter construct and a gene with an unknown impact on motility are coupled with biochemical assays to quantitate the number of cells that have received a transferred gene, which subsequently crosses the membrane. This assay was found to be less variable than the conventional transwell chamber and is easily adaptable to studies of cell motility or cell invasion. We also demonstrate that this assay can detect the effect of both genetic and pharmacological inhibition of motility alone and in combination. It therefore has the potential to reveal additive or synergistic effects.

INTRODUCTION

A tumor’s transition from a hyperproliferative to an invasive lesion is associated with a significant decrease in patient survival. A window of opportunity thus exists before the invasive disease develops. This has resulted in significant efforts to develop novel therapies that delay or prevent such an invasion. Directed tumor cell motility or chemotaxis is the final step of tumor invasion, and the inhibition of this process has been a major focus of research. Several modifications of the transwell assay have been used to quantitate both the invasive and chemotactic response of cells. In the classic assay,