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Quantification of nucleic acids on nitrocellulose membranes with time-resolved fluorometry

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ABSTRACT

We use a streptavidin-based macromolecular complex (SBMC) labelled with the europium chelate of 4,7-bis (chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) as a staining reagent for biotinylated DNA present on nitrocellulose filters. The fluorescent spots or bands obtained can either be observed under UV illumination, photographed by instant camera photography or quantified by using a specially designed instrument working as a high resolution time-resolved fluorometric scanner. The detection limit is ~10 pg of target DNA. Various experiments with use of biotinylated DNA probes hybridized to Southern transferred targets have shown that the new procedure is a useful versatile non-isotopic methodology for staining DNA on solid supports.

INTRODUCTION

Molecular biology techniques are still not widely used for routine clinical diagnosis partly because they rely on the use of radioactive nuclides. This problem has been resolved in the field of immunological assays where non-isotopic methodologies are now very well-established (1, 2). Non-isotopic nucleic acid hybridization assays have been reported and are increasingly used successfully for three reasons (a) some of the newer techniques are very sensitive, approaching the detectability of assays based on ^{32}P (3, 4); (b) for certain applications, extreme sensitivity is not needed and (c) with the advent of the polymerase chain reaction (PCR), low abundance targets can be amplified before quantification (5, 6).

The techniques now used for non-isotopic nucleic acid hybridization assays are similar to those used for non-isotopic immunological assays and include the use of enzymes, luminescent and fluorescent labels (7, 8). Time-resolved fluorometry, with europium chelates as labels, is now an established technique in the field of non-isotopic immunoassay (9, 10). The numerous advantages of the fluorescent Eu^{3+}-chelates over conventional fluorescent probes have been summarized elsewhere (8). In one assay design using such chelates, the reactants (antibodies or DNA probes) are labelled with Eu^{3+} (11). These reactants are not fluorescent and in order to quantify Eu^{3+} with high sensitivity, Eu^{3+} must be extracted from the target-reactant complex and re-complexed in solution with appropriate organic ligands. This approach is successful for immunological assays and some nucleic acid hybridization assays where electrophoretic separation and blotting is not used (12–14). However, Eu^{3+} labelling is not suitable for the widely used techniques of Southern, Northern and Western blotting because during the extraction of Eu^{3+} from the target-labelled reactant complex, the spatial distribution of Eu^{3+} is lost.

We have recently described a streptavidin-based macromolecular complex (SBMC) which is multiply labelled with the europium chelate of 4,7-bis (chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) (15, 16). SBMC is represented by the empirical formula $SA(TG)_{3.3}$ $(BCPDA)_{480}$, where SA stands for streptavidin and TG for bovine thyroglobulin. The reagent is saturated with Eu^{3+} which forms fluorescent complexes with BCPDA. In this complex, Eu^{3+} is entrapped within the core of the complex and is quantitatively retained during the detection procedure. SBMC is stable for at least one year and it has already been used successfully in a number of immunological assays where biotinylated antibodies are employed as complementary reagents (17, 18). In such assays, solid-phase (polystyrene microtitration wells) time-resolved fluorometric measurements are performed for quantification, using a specially designed time-resolved fluorometer. In this report, we describe the application of the SBMC for nucleic acid quantification on nitrocellulose membranes. In all applications, the nucleic acids are either biotinylated or hybridized to biotinylated probes in typical Southern blotting experiments. The final fluorescent complex is visible under UV light, can be photographed with conventional instant cameras and can also be quantified by using a modified time-resolved fluorometer working as a high resolution scanning device. The combination of the scanner and the SBMC constitutes a new quantitative and sensitive system which could find many applications in the field of biotechnology.

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MATERIALS AND METHODS

Instrumentation

The CyberFluor 615™ immunoanalyzer is available from CyberFluor Inc. Toronto, Canada. A special software was written which transforms this instrument from a solid-phase microplate time-resolved fluorescence reader to a high resolution time-resolved fluorescence scanning device, as described later in the text. The special software is also available through CyberFluor.

Materials

The streptavidin-based macromolecular complex (SBMC) was prepared as previously described (16) and is commercially available from CyberFluor Inc. The stock solution, containing 15 mg/L of streptavidin was diluted just before use, 50-fold, in a diluent which is a 50 mM Tris buffer, pH 7.20, containing 40 g of bovine serum albumin (BSA), 0.5 mol of KCl, 0.15 mol of NaCl, 4×10^{-5} mol of $EuCl_3$ and 0.5 g of NaN_3 per liter.

Biotinylated DNA molecular weight markers (HindIII lambda DNA digests) were obtained from Vector Laboratories, Burlingame, CA 94010, as a 50 μg/mL solution.

Purified linearized plasmid pBR328 and its digestion fragments were obtained from Boehringer-Mannheim, Indianapolis, IN 46250 as part of their 'DNA labelling and detection kit, nonradioactive'. Linearized plasmid pBR328 was biotinylated using the random primer method with reagents and biotin 11-dUTP purchased from Stratagene (Prim-it™ Random Primer Kit supplemented with Bio-11-dUTP/dTTP mix).

Nitrocellulose membranes were obtained from Amersham (supported nitrocellulose Hybond-C-Extra).

Evaluation of instrument's performance

A detailed description of the time-resolved fluorometric scanner follows in the text. The instrument's performance was evaluated by using the SBMC at various dilutions in the tracer diluent. Spots of diluted SBMC (0.5 or 1 μL) were placed on either white opaque 'U' shape plates (from Dynatech Labs, Alexandria, VA) or nitrocellulose and scanned.

Detection of biotinylated nucleic acids on spots

The stock 50 μg/ml biotinylated nucleic acid markers solution (Vector) was serially diluted in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.80) containing salmon sperm DNA (Sigma) at a concentration of 400 μg/mL. In Eppendorf microcentrifuge tubes, we added 10 μL of diluted biotinylated DNA and 10 μL of denaturation buffer (prepared by mixing 300 mL of 5 M NaCl, 50 mL of 10 M NaOH and 650 mL of H_2O) and incubated for 10 min at room temperature. We then added 20 μl of neutralization buffer (5 M ammonium acetate solution) mixed and spotted 1 μL on prewetted (50 mM Tris, pH 7.40) nitrocellulose strips. The strips were baked for 1 h at 60°C and then blocked in 6% BSA solution (in a 50 mM Tris buffer, pH 7.80) for 1 h to overnight. The biotinylated nucleic acids were visualized with 50-fold diluted SBMC in tracer diluent for 3 h at RT with continuous rotational shaking. At the end of the incubation, the strips were washed ×3 with a wash solution (50 mM Tris, pH 7.20 containing 0.05% Tween 20 and 9 g/L of NACl) and soaked with shaking for 1 h in the same solution. The strips were then dried with a hair-dryer and observed under UV light (spotted side down) and scanned with the instrument as described later (spotted side up). The strips could also be photographed under UV light using a Polaroid camera under the same conditions used for photographing ethidium bromide stained agarose gels, with an exposure time of ~13 s.

Southern transfer of biotinylated markers

Biotinylated DNA markers were separated with agarose gel electrophoresis and Southern transferred to nitrocellulose using standard procedures (19). The membrane was then baked for 2 h at 80°C, blocked with 6% BSA, stained with the SBMC and washed exactly as described under detection of biotinylated nucleic acids on spots.

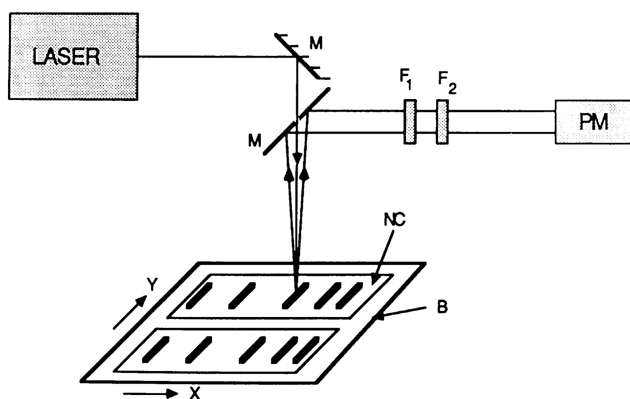

Figure 1. Simplified optical diagram of the CyberFluor 615™ Immunoanalyzer working as a high resolution time-resolved fluorometric scanner. The excitation source is a nitrogen laser emitting 20 pulses of light (337.1 nm) per s, lasting ~3 ns. M = mirrors, one with a hole in the middle; F1, F2 are liquid and interference filters, respectively. Other lenses, mirrors and filters and other components have been eliminated for simplicity. The electronic circuitry used for the time-resolved measurement is not shown. The metal base (B) used to position the nitrocellulose membranes (NC) is an exact replica of a microtitration plate. The metal base movement is in the × and Y direction as shown and also described in the text. Components are not shown to scale. For more information, see text and Reference 9.

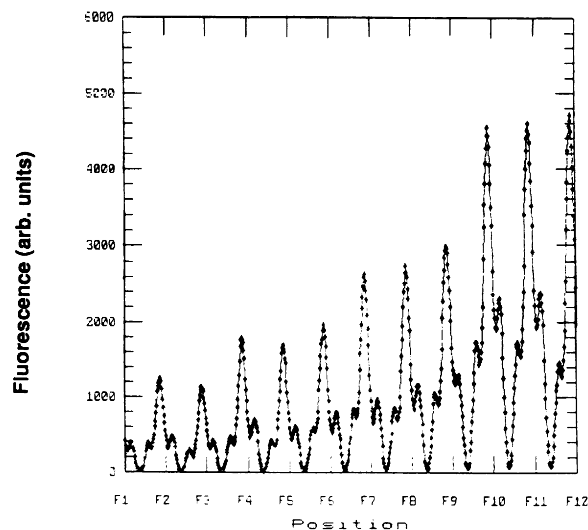

Figure 2. Quantification of the SBMC present in white opaque 'U' shape microtitration wells (1 μL/well). The wells were scanned from F1−F12. The shape of the peaks is not smooth because of the uneven shape of the 1 μL drop. The amount of the SBMC complex (in moles) is: 9.8×10^{-18} (F1−F3); 2.0×10^{-17} (F4−F6); 3.9×10^{-17} (F7−F9) and 7.8×10^{-17} (F10−F12). Scanning points used were 700.

Hybridization experiments

Nucleic acid targets were separated with agarose gel electrophoresis and transferred to nitrocellulose using standard procedures (19). The membranes were then baked for 2 h at 80°C and prehybridized in the prehybridization buffer (5×SSPE, 5×Denhardts solution, 0.1% SDS, 100 μg/mL denatured salmon sperm DNA) for 2 h at 65°C. The 20×SSPE solution contains per liter 174 g NaCl, 27.6 g $NaH_2PO_4 \cdot H_2O$, 7.4 g EDTA, pH adjusted to 7.4 with NaOH. The 100×Denhardts solution is 2% polyvinylpyrrolidone, 2% bovine serum albumin, 2% Ficoll. Hybridizations were performed in the hybridization buffer (exactly as the prehybridization buffer but with 1×Denhardts) at 65°C, overnight. Both prehybridizations and hybridizations were performed in 20 mL solutions using the Robbins Scientific hybridization incubator. Biotinylated probes were boiled for 5 min before they are added to the hybridization solution at a concentration of 10 ng/mL.

After hybridization, the membranes were washed as follows: Three times with 2×SSC, 0.1% SDS at room temperature (RT), 5 min each, with vigorous shaking (20×SSC is 175.3 g NaCl, 88.2 g trisodium citrate · 2 H_2O, pH adjusted to 7.0 with HCl and volume adjusted to 1 L). Three times as above but with 0.2×SSC, 0.1% SDS. Two times with 0.2×SSC, 0.1% SDS at 65°C, 15 min each.

After blocking with 6% BSA, the membranes were stained with the SBMC and washed exactly as described under detection of biotinylated nucleic acids on spots.

RESULTS AND DISCUSSION

Instrument description

A brief description of the CyberFluor 615™ Immunoanalyzer has been published elsewhere (9). This instrument is a time-resolved fluorometer optimized for measuring delayed fluorescence emitted from europium chelates immobilized on dry solid-phases like polystyrene microtitration wells. The excitation source is a nitrogen laser (337.1 nm). The instrument measures fluorescence on a certain spot on the solid-phase, twenty times, and uses the sixteen replicates to obtain a mean fluorescence value. One mean value is generated in only 1 s. For multiple well measurements the instrument moves the microtitration wells on an X−Y direction. The standard microtitration plate configuration has 8 rows, A, B, C, D, E, F, G, H; each row has 12-wells. The 96 wells of the plate are measured by the instrument as follows: After the plate is positioned in the optical pathway (Figure 1), the first well to be measured is A1 followed by A2 .... A12 as the plate moves in the X-direction, one well at a time (1 s per well). Then, the plate moves to the Y-direction so that B1 is the new starting well and so on.

We have altered the standard software of the above instrument (standard and new software can be interchanged in a few minutes by loading it through diskettes) so that the standard movement from A1 to A12 or any other row is modified as follows: In the standard software the movement takes place from the centre of one well to the centre of the next (distance 0.91 cm). In the new software, the instrument can move the plate from the middle of A1 to the middle of A12 (distance 10 cm) in a selectable increment which ranges from 12 to 770. That is, each step can be as short as 0.0129 cm, which is the maximum spatial resolution of the instrument. With the new software, the operator has the ability to chose which row of the plate to scan (A to H), the starting well and the finishing well of the row (starting and finishing well of the row can be any well between 1 and 12) and the number of measurements to be performed (between 12 and 770). For scanning one row, we usually select 100 measurement points for preliminary information and up to 770 measurement

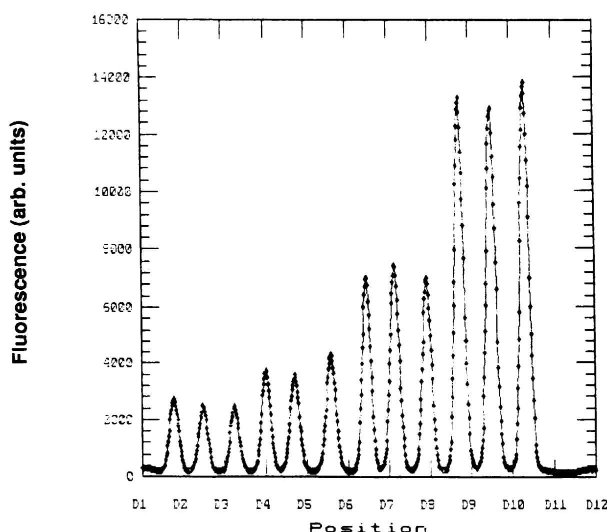

Figure 3. Quantification of the SBMC present on nitrocellulose spots (0.5 μL/spot; triplicate applications). The amount of the SBMC (in moles) is: 7.8×10^{-17} (D1−D3); 1.6×10^{-16} (D4−D6); 3.1×10^{-16} (D6−D8); 6.2×10^{-16} (D9−D11). Scanning points used were 700.

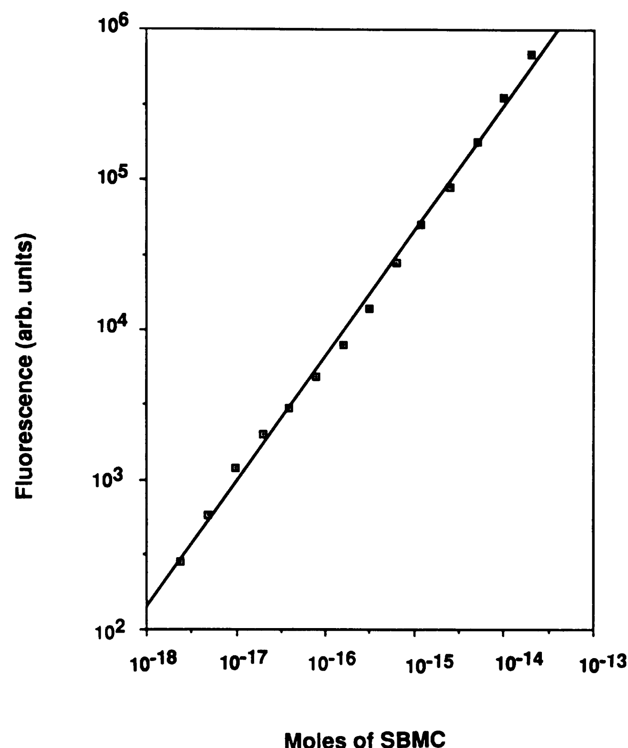

Figure 4. Double logarithmic plot of fluorescence (peak height) vs moles of the SBMC. The SBMC was spotted in white opaque 'U' shape microtitration wells (1 μL/well) and scanned as shown in Figure 2. Linearity extends from the detection limit to ~10^{-14} moles of SBMC.

points for maximum resolution. Scanning time varies according to the measurement points (1 s/point).

The data collected by the instrument can be presented graphically (fluorescence vs distance) and the plot, from the instrument's screen, can be printed with or without the raw fluorescence data. We have constructed a metal base which is a replica of a standard microtitration plate, on which we mount the nitrocellulose strips to be scanned by using adhesive tape.

Evaluation of the instrumental sensitivity

The SBMC was diluted successively and then spotted on white opaque microtitration plate wells (1 μL) or nitrocellulose filters (0.5 μL). The spots were then scanned on the instrument. Some results are shown in Figures 2 and 3. The instrument can reliably discriminate down to about 6×10^{-19} moles of the SBMC (signal with this SBMC amount is 2-fold greater than the signal obtained with tracer diluent only). This amount of the SBMC is equivalent to $\sim$360,000 molecules of the streptavidin conjugate. The linearity of response extends from the detection limit to approximately 10^{-14} moles of the SBMC (approximately 4 decades) (Figure 4).

Detection of biotinylated nucleic acids on spots

Varying amounts of biotinylated DNA (lambda DNA digested with HindIII and labelled with photobiotin) were spotted on nitrocellulose and visualized with the SBMC (1 μL spots). Some results are shown in Figure 5. Visually, under UV transillumination, spots containing 400 pg or more of DNA can be easily seen. With instrumental scanning, the detection limit of biotinylated DNA was $\leq$ 10 pg (1 μL spots). The response of the instrument was nearly linearly related to the amount of spotted DNA. The instrument can zoom on any of the spots if necessary, for better resolution (Figure 5c).

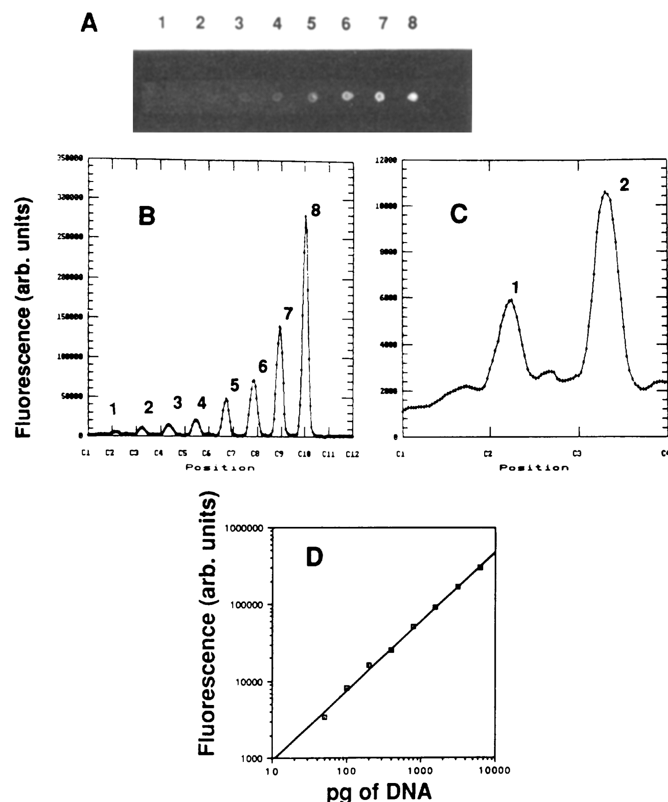

Figure 5. Detection of biotinylated lambda DNA on spots with the SBMC (1 μL/spot). (**A**) Photograph of the spots under UV transillumination; (**B**) Scanning of the spots with the modified 615™ Immunoanalyzer (200 measurement points); (**C**) Scanning of positions C1–C4 of Figure (B) to improve resolution and (**D**) Double logarithmic plot of fluorescence vs amount of biotinylated DNA spotted. Biotinylated DNA spotted (pg/1 μL) was 50(1); 100(2); 200(3); 400(4); 800(5); 1600(6); 3200(7); 6400(8).

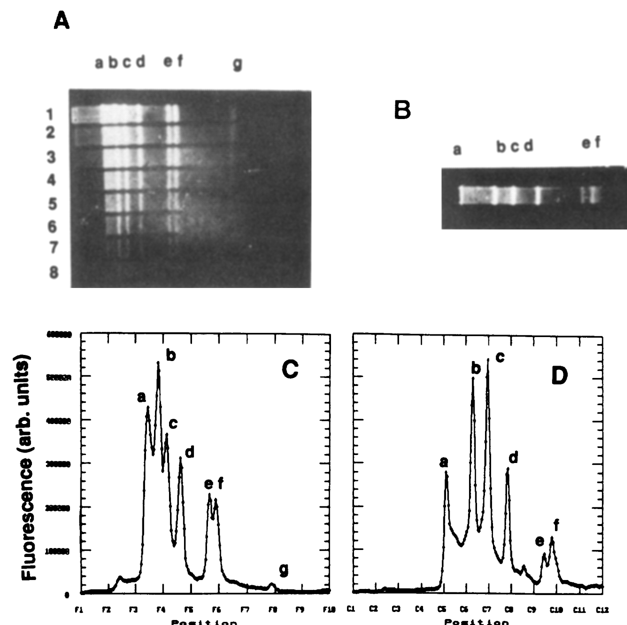

Figure 6. Transfer of biotinylated DNA markers from agarose to nitrocellulose and staining with the SBMC. (**A**) Amount of DNA loaded on a 1% agarose gel was (in ng): 500(1); 250(2); 125(3); 62(4); 31(5); 16(6); 8(7); 4(8); (**B**) Amount of DNA loaded on a 0.7% agarose gel was 250 ng; (**C**) and (**D**) represent instrument scans (200 points) of A (250 ng lane) and B, respectively. The length of the fragments (Kb) are 23.1 (a); 9.4(b); 6.7(c); 4.4(d); 2.3(e); 2.0(f); 0.56(g).

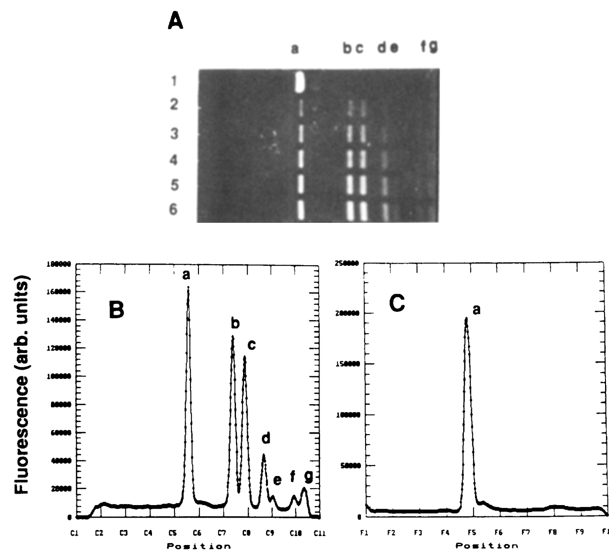

Figure 7. (A) Hybridization experiments after Southern blotting, of various amounts of enzyme-digested (lanes 2–6) or undigested (lane 1) linearized plamid pBR328 from 0.8% agarose gels. The probe was biotinylated linearized pBR328 plasmid. The total amount of DNA (in ng) in lanes, was 160(1); 5(2); 10(3); 20(4); 40(5); 80(6). The length of each fragment is (in base pairs) 4907(a); 2176(b); 1766(c); 1230(d); 1033(e); 653(f); 517(g). (B) Time-resolved fluorometric scanning of lane 6 (700 points) indicating the seven bands. (C) as in (B) but for lane 1.

Southern transfer of biotinylated markers

Biotinylated HindIII lambda DNA markers were separated by electrophoresis on 1% or 0.7% agarose gels and Southern transferred to nitrocellulose. The markers were then stained with the SBMC as described. The results are shown in Figure 6. As low as 4 ng of markers can be seen and the obtained pattern is identical to that obtained by using streptavidin-alkaline phosphatase conjugates and the BCIP-NBT substrate (data not shown).

Hybridization experiments

Plasmid pBR328 was digested with restriction endonucleases. Then, varying amounts were subjected to 0.8% agarose gel electrophoresis along with the undigested plasmid and subsequently transferred to nitrocellulose. After hybridization with biotinylated linearized plasmid pBR328 the membrane was stained with SBMC as described. Some results are summarized in Figure 7.

We have presented data to show that a new streptavidin-based macromolecular complex multiply labelled with a europium chelate, is a suitable reagent for the quantification of nucleic acids on nitrocellulose membranes with use of time-resolved fluorometry. The limitation of the system in its present form is its inferior sensitivity in comparison to some other non-isotopic nucleic acid detection systems (3, 4). The advantages of this new system are as follows: (a) Non-isotopic detection; (b) excellent reagent stability (> 1 year); (c) no need for autoradiography or enzyme substrates; (d) signal on nitrocellulose is stable for months to years and visible on UV-illuminators with the possibility of performing standard instant photography if desired and (e) with use of the high resolution scanning time-resolved fluorometer the system can quantify the fluorescent bands and (f) the new system is more sensitive and has a much wider range of applications than different time-resolved fluorometric methods used for nucleic acid applications based on Eu^{3+} labelling (12–14). The latter methods are not suitable for quantifying nucleic acids transferred to solid supports because they rely on Eu^{3+} extraction.

Recently, another time-resolved fluorescence method for DNA detection was described (21) where a double stranded poly (A-T) tail was initially attached to the end of an oligonucleotide probe and then crosslinked by psoralen molecules. Then, fluorescent Tb^{3+}-DTPA-pAS chelates (where DTPA is diethylenetriamine pentaacetic acid and pAS is p-aminosalicylic acid) were attached to psoralen amino groups and can be detected with time-resolved fluorometry. We feel that the present method is advantageous because it is simpler (incorporation of biotinylated nucleotides is an established procedure) and it can be applied to any biotinylated nucleic acid probe independently of the labelling position without any loss in sensitivity. Work is now in progress to apply the method in other areas of biotechnology.

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