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Evaluation of Nonisotopic Labeling and Detection Techniques for Nucleic Acid Hybridization

Eleftherios P. Diamandis,[1,2] Stavroula Hassapoglidou,[1] and Courtney C. Bean[1]

[1]Department of Clinical Biochemistry, Toronto Hospital, Toronto Western Division, Toronto, Ontario, Canada;
[2]Department of Clinical Biochemistry, University of Toronto, Toronto, Ontario, Canada

We have used a double-stranded DNA probe linked to the cystic fibrosis locus to detect a single-copy gene from varying amounts of genomic DNA, with Southern blot analysis. The DNA plasmid probe was labeled with either biotin or digoxigenin. Biotin or digoxigenin was then linked to alkaline phosphatase (ALP) with use of streptavidin or anti-digoxigenin antibodies, respectively. ALP activity was then detected with a chromogenic (BCIP/NBT), chemiluminogenic (AMPPD), or fluorogenic (HNPP) substrate. Our results suggest that biotin and digoxigenin perform similarly and that the three substrates exhibit similar detectability under appropriate substrate incubation times: 20–120 min (AMPPD), 2–12 h (HNPP), and 18–48 h (BCIP/NBT). Under optimised conditions and the probe used, these methods detect single-copy genes from as little as 0.3 μg of total genomic DNA.

Key words: DNA probes, chemiluminescence, Southern blot, biotin, digoxigenin, nonisotopic detection

INTRODUCTION

Nucleic acid hybridization is a powerful technique that is now used frequently for research purposes and routine testing. Some of the current applications include the detection of infectious agents; the study and diagnosis of genetic, malignant, or hematologic disease; and the identification of individuals in forensics (1–3). Routine testing with nucleic acid hybridization techniques is growing at a relatively slow pace because the current methods are cumbersome, time-consuming, and relatively expensive. Also, until recently, the label of choice for such assays was the radionuclide ^{32}P, which has a short half-life; it poses disposal and safety problems and necessitates long exposure times of many hours to many days.

Clearly, the transition of nucleic acid hybridization assays from the research to the routine laboratory will be greatly facilitated by the simplification of the current assay formats, the introduction of at least some automation, and the use of nonisotopic labeling and detection systems. Recently, a number of nonisotopic labeling and detection systems have been proposed for nucleic acid hybridization and are reviewed elsewhere (4,5). It is claimed that some of these systems are as sensitive as the ^{32}P-based assays. In this paper, we evaluate the ability of some nonisotopic labeling and detection systems to detect single-copy genes on Southern blots from varying amounts of human genomic DNA. Under optimized conditions, it is shown that with the systems evaluated, single copy genes can be detected from as little as 0.3 μg of total genomic DNA.

MATERIALS AND METHODS

Materials

The restriction endonuclease *Hind*III, approximately 50 U/μl, and digoxigenin-labeled DNA molecular-weight markers II, were from Boehringer Mannheim (Laval, PQ, Canada). The DNA labeling and detection kit nonradioactive containing all the reagents necessary to label DNA with digoxigenin by the random primer method and detect the DIG-labeled DNA with an alkaline phosphatase-conjugated anti-DIG antibody, was also from Boehringer. The Photogene nucleic acid detection system containing all the reagents necessary to detect the biotinylated DNA with an alkaline phosphatase-conjugated streptavidin, was from BRL Life Technologies (Gaithersburg, MD 20877). Biotinylated probe was prepared using the BioNick labeling system, which labels double-stranded DNA by nick translation. This kit was also obtained from BRL.

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Address reprint requests to Dr. Eleftherios P. Diamandis, Department of Clinical Biochemistry, Toronto Hospital, Toronto Western Division, 399 Bathurst Street. Toronto, Ontario M5T 2S8, Canada.

Nonstandard Abbreviations: DIG, digoxigenin; AMPPD, adamandyl-1,2-dioxetane phosphate; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; HNPP, 2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate; SDS, sodium dodecyl sulfate; Kb, kilobase; ALP, alkaline phosphatase.

DNA was extracted from EDTA-anticoagulated human blood with the method of Miller et al. (6). The probe used, 7C22, was obtained as purified plasmid from the American Type Culture Collection, (Rockville, MD; Cat. no. 57545). Agarose was from FMC BioProducts (Rockland, ME). The biotinylated DNA molecular-weight markers were from Vector Laboratories (Burlingame, CA). Nylon membranes, Hybond-N, were from Amersham International (Buckinghamshire, England) and the X-OMAT-S X-ray films from Eastman Kodak (Rochester, NY 14650). The alkaline phosphatase substrate HNPP was a gift from AISIN Seiki Co. (Tokyo, Japan). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Solutions

The 20× SSC is a 3.0-mol/L NaCl and a 0.3-mol/L sodium citrate solution of pH 7.0. More dilute solutions were prepared by dilution of this stock. The prehybridization solution contains the following: formamide, 50% (v/v); NaCl, 0.9 mol/L; sodium phosphate, 0.06 mol/L; EDTA, 0.006 mol/L; Ficoll, 0.1% (w/v); polyvinylpyrrolidone, 0.1% (w/v); bovine serum albumin (BSA) 0.1% (w/v); sodium dodecyl sulfate (SDS), 1% (w/v); and sheared denatured salmon sperm DNA, 200 μg/ml. For a 200-ml solution, the solid components of this mixture except DNA were dissolved in 40 ml of deionized water, pH adjusted to 7.4 with 4 mol/L NaOH; the salmon sperm DNA was then added (2 ml of a 10-mg/ml stock solution). The volume was then adjusted to 50 ml; 50 ml of formamide was added. This solution is stored at −20°C. A 20% (w/v) dextran sulfate solution was prepared by dissolving 10 g of dextran sulfate in 50 ml formamide, with continuous stirring overnight, and was stored at 4°C. The 2× hybridization solution contains NaCl, 1.8 mol/L; sodium phosphate, 0.12 mol/L; EDTA, 0.012 mol/L; Ficoll, 0.2% (w/v); polyvinylpyrrolidone, 0.2% (w/v); BSA, 0.2% (w/v); SDS, 2% (w/v), and sheared denatured salmon sperm DNA, 400 μg/ml. To make a 50-ml solution, the solid components of the mixture, except DNA, were dissolved in 40 ml of deionized water and pH adjusted to 7.4 with 4 mol/L NaOH. The salmon sperm DNA was then added (2 ml) and the final volume adjusted to 50 ml. The final hybridization solution is prepared by mixing equal volumes of 20% dextran sulfate solution in formamide and 2× hybridization solution. The TBS-Tween 20 solution is 100 mmol/L Tris, 150 mmol/L NaCl, and 0.05% (v/v) Tween 20, having a final pH of 7.5. The blocking solution for the biotin-labeled probe system is a 3% (w/v) BSA solution in the TBS–Tween 20 buffer. The blocking solution for the digoxigenin-labeled probe system, provided in the kit, was prepared as suggested by Boehringer.

Probe

The probe used, 7C22, is a pSP64 plasmid with a cloned genomic insert of 5.10 kb. The insert binds to a locus linked to the locus of cystic fibrosis (7,8). The purified plasmid, obtained from ATCC, was biotin-labeled by nick-translation without further treatment, following the instructions of the kit. The plasmid was linearized with *Hind*III as described elsewhere (9) and was digoxigenin-labeled by the random primer method, following the instructions of the kit.

This probe hybridizes to a *Hind*III fragment of human genomic DNA of 8.1 kb approximate size (8).

Methods

Extraction of human DNA from leukocytes, digestion, and Southern blot

We used 10 ml of blood collected from healthy volunteers in EDTA-containing blood collection tubes. Extraction of DNA from leucocytes was performed according to the procedure of Miller et al. (6). The obtained DNA (approximately 100–200 μg, $A_{260}/A_{280} > 1.70$, $M_r > 20$ kb) was digested with *Hind*III, using standard procedures (9). Digestion was stopped by heating at 65°C for 10 min. Before electrophoresis, the digested DNA was heated for 10 min at 65°C to separate cohesive terminii and then stored on ice until loading. Electrophoresis was performed on 0.8% agarose mini-gels in Tris–acetate–EDTA buffer (9) at 3 V/cm. After electrophoresis (5–6 h), the gel was Southern-blotted overnight using the protocol and solutions described by Davis et al. (10). The nylon membrane was then baked in a vacuum oven at 80°C for 2 h to fix the single-stranded DNA.

Preparation of labeled probe

The plasmid probe was biotin-labeled with the nick-translation method using the BioNick kit according to the manufacturer's instructions. With this method, biotin-14-dATP is incorporated into double-stranded DNA. The plasmid probe was digoxigenin-labeled with the random primer method, using a kit from Boehringer, according to the manufacturer's instructions. With this method, the plasmid is first linearized with *Hind*III. During labeling, digoxigenin − 11 — dUTP is incorporated into double-stranded DNA. Labeled probes were purified from unincorporated nucleotides using disposable PD-10 Sephadex columns (Pharmacia) and an in-house protocol as follows. Wash column with 10 ml of a Tris–EDTA buffer (TE) (9) and let it drain. Apply the labeling mixture (50 μl) and let it enter the gel. Wash column with exactly 2 × 1 ml of TE buffer, and discard eluate. To elute probe add 3 × 0.5 ml of TE buffer and collect eluate. The final eluate (1.5 ml) has a probe concentration of approximately 660 ng/ml when 1 μg plasmid is used for labeling.

Hybridization and blocking

The hybridization protocol for the biotin system was as follows. Prehybridization of the membrane in 17 ml of prehybridization solution for 4 h at 42°C, in a hybridization chamber

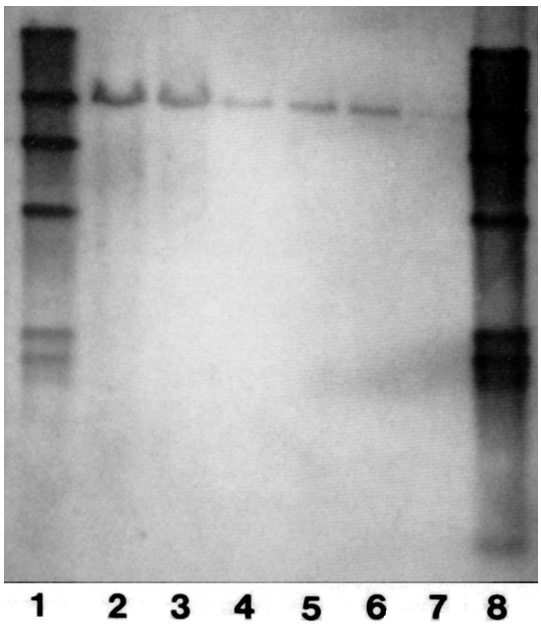

Fig. 1. Chemiluminescent detection of human genomic DNA digested with *Hind*III, after hybridization with a biotin-labeled probe. For details see text. **Lanes 1 and 8**, biotinylated molecular-weight markers (lambda DNA digested with *Hind*III). Loadings were 1 ng **(lane 1)** and 4 ng **(lane 8).** The molecular weight of the marker bands are 23.1, 9.4, 6.6, 4.3, 2.2, and 2.0 kb. The genomic DNA loadings were 10, 5, 2.5, 1.2, 0.6, and 0.3 μg, in lanes 2, 3, 4, 5, 6 and 7, respectively. Exposure time was 30 min.

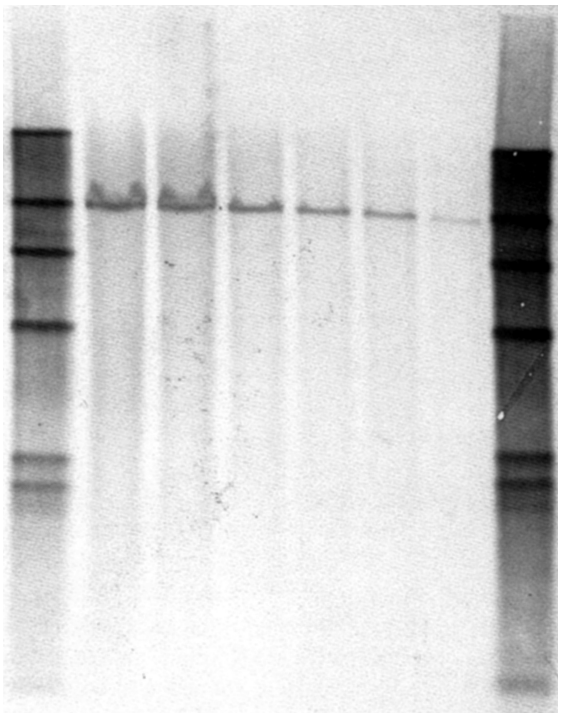

Fig. 2. Colorimetric detection of human genomic DNA digested with *Hind*III, after hybridization with a biotin-labeled probe. Other information is the same as in Figure 1. Substrate incubation was for 18 h.

equipped with glass tubes (from BioCan Scientific, Toronto, ON). Hybridization overnight (approximately 16 h) in 5.2 ml of hybridization solution, containing heat-denatured labeled probe (approximately 30–40 ng/ml final concentration) at 42°C. The membrane was then removed and washed as follows: once at room temperature with wash solution A (2 × SSC, 0.1% SDS) for 5 min, followed by 3 × 15-min washes; and twice at 50°C with wash solution B (0.1 × SSC, 0.1% SDS), 15 min each. The membrane is then wetted in TBS–Tween 20 solution for 1 min and blocked by incubation in 50 ml of blocking solution at 65°C for 1 h. Hybridization with the digoxigenin system was identical, except for the following. The probe concentration was around 20 ng/ml; washing with solution B was at 65°C; and blocking was performed with the blocking reagent provided by Boehringer and the protocol suggested.

Detection with the biotin system

Dilute 40 μl of a streptavidin–alkaline phosphatase conjugate (1 mg/ml, BRL) 1,000-fold in the TBS-Tween 20 diluent. Incubate the membrane with the solution for 10 min, with gentle agitation. Membrane is then washed twice with 60–70 ml of TBS–Tween, 15 min each, at room temperature. The membrane is finally washed for 1 h at room temperature with a "final wash buffer" provided with the Photogene kit and blotted dry on Whatman 3MM filter paper. The membrane is then placed into a plastic development folder (included in the BRL kit) and 700 μl of AMPPD detection solution is layered on top. After closing the top plastic cover, a pipet is rolled over to ensure even distribution of the reagent. The plastic folder is then heat-sealed and stored in the dark for about 1 h, before exposure onto an X-ray film. Exposure time was optimized empirically to achieve high sensitivity and low background and varied from 20 to 120 min. The same membrane can be used for either detection with

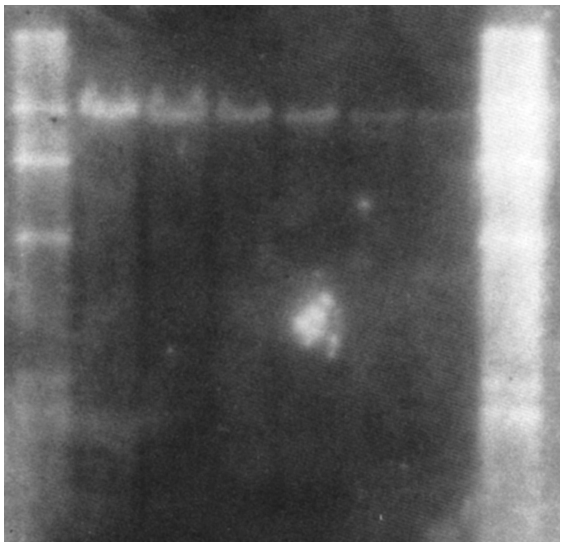

Fig. 3. Fluorometric detection of human genomic DNA digested with *Hind*III, after hybridization with a biotin-labeled probe. Other information is the same as in Figure 1. Substrate incubation was for 8 h.

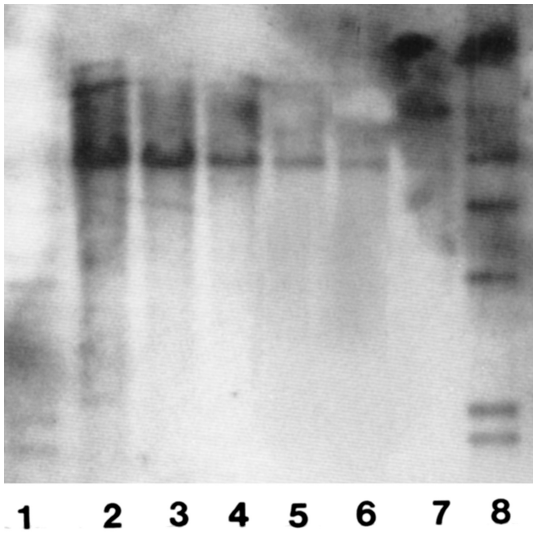

Fig. 4. Chemiluminescent detection of human genomic DNA digested with *Hind*III, after hybridization with a digoxigenin-labeled probe. For details see text. **Lanes 1 and 8**, digoxigenin-labeled molecular-weight markers (lambda DNA digested *Hind*III). Loadings were 1 ng **(lane 1)** and 4 ng **(lane 8)**. Other conditions as in Figure 1. Exposure time was 20 min.

the BCIP/NBT method or the HNPP method, as follows. The AMPPD reagent is removed from the membrane with two 5-min washes, with 50 ml of buffer 3 (Tris, 0.1 mol/L, pH 9.5, containing 0.1 mol/L NaCl and 50 mmol/L $MgCl_2$). The membrane is then incubated in 20 ml of a BCIP/NBT solution (prepared as described in the Boehringer kit) for 18–48 h in the dark. After incubation, the membrane is washed with 50 mL of buffer 4 (Tris, 10 mmol/L, pH 8.0, containing 1 mmol/L EDTA) for 5 min. The result is documented by photocopying the dry membrane. For detection with the HNPP reagent, the membrane is incubated with 40 ml of HNPP working solution (a 400-fold diluted stock solution, 20 mg/mL in dimethylformamide, in buffer 3) for 2–12 h at room temperature in the dark. The membrane is then removed and placed on top of a Whatman 3MM paper, which was soaked with a 0.5-mol/L NaOH solution. The same NaOH solution was also layered on top; results were documented by photography with a Polaroid camera under UV irradiation as described elsewhere (11).

Detection with the digoxigenin system

The blocked membrane was incubated with 50 ml of a 5000-fold diluted anti-digoxigenin antibody conjugated to alkaline phosphatase. The diluent was the blocking solution. Unbound antibody conjugate was removed by washing the membrane twice, 15 min each, with 100 mL of buffer 1 (100 mmol/L, maleic acid, 150 mmol/L NaCl, pH 7.50). After washing, the membrane is equilibrated for 2 min in buffer 3 and excess liquid is blotted out with filter paper. Detection with the reagents described before, is performed identically as described for the biotin-labeled probes.

RESULTS AND DISCUSSION

Hybridization experiments with use of biotin-labeled probes and final detection with AMPPD, BCIP/NBT, and HNPP are shown in Figures 1, 2, and 3, respectively. In these experi-

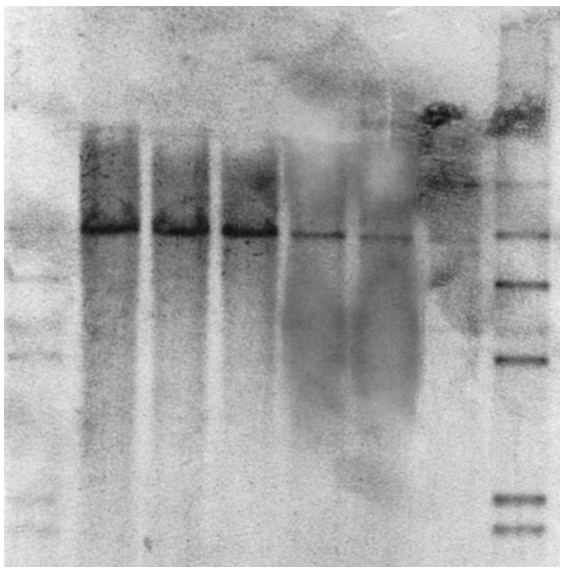

Fig. 5. Colorimetric detection of human genomic DNA digested with *Hind*III, after hybridization with a digoxigenin-labeled probe. Other information is the same as in Figure 4. Substrate incubation was for 16 h.

ments, variable amounts of total genomic DNA, digested with *Hind*III, were loaded in order to assess the detection limit of each technique. Visible hybridization bands were observed even with loadings of 0.3 μg (lane 7). Additional data, with digoxigenin-labeled probe and the detection methodology of AMPPD and BCIP/NBT are shown in Figures 4 and 5. Similarly, hybridization bands were visible up to loadings of 0.3 μg of total genomic DNA. In all cases, the bands were sharp and corresponded to the expected molecular weight as previously reported with radioactive labeling and the same probe (7,8). The major difference between the three detection methodologies, is the length of time needed to generate enough signal. Optimal incubation times with the substrate and/or exposure times were somewhat variable and need to be optimised for each experiment. In general, these timings were within 20–120 min (AMPPD), 2–12 h (HNPP), and 18–48 h (BCIP/NBT).

Nucleic acid hybridization has the potential to markedly improve the routine diagnosis of infectious, genetic and malignant disease. In order for these tests to become routine, nonisotopic detection method must be utilised without compromising sensitivity. Radioactive nuclides, although widely used in research, are unsuitable for mass testing due to concerns of hazards and disposal. A number of routine nucleic acid hybridization tests based on nonisotopic detection are now commercially available (1,12).

The current major nonisotopic nucleic acid hybridization assays are based on chemiluminescence, fluorescence or colorimetric detection (4,13–15). The chemiluminescent techniques are based mostly on acridinium ester methodology, enzymatically triggered chemiluminescent substrates or enhanced luminescence (4). The first two technologies have recently been reviewed and compared (12). There are many other reports in the literature which confirm the excellent sensitivity of the AMPPD substrate (13–15) in agreement with the findings of this study. Direct comparison of the AMPPD and BCIP/NBT methods by using the same hybridization membrane (Figs. 1, 2, 4, 5) reveals the similar detectability of the two methods when optimized incubation times are used. In general, biotin or digoxigenin labeling give comparable results.

Recently, Kagiyama et al. (11) introduced a new alkaline phosphatase substrate, HNPP, suitable for nucleic acid hybridization. The new substrate has affinity for nylon when dephosphorylated by ALP. The fluorescent product can be photographed under UV light to obtain permanent records of the results. HNPP has not been previously used to detect human DNA on Southern blots. We have shown that with this substrate it is possible to detect single-copy genes from as low as 0.3 μg of human total genomic DNA (see Fig. 3). In this respect, HNPP has similar detectability to AMPDD and BCIP/NBT. A distinct advantage of this substrate in comparison to BCIP/NBT is that reprobing is possible because the fluorescent product can be easily removed from nylon by a simple wash with dimethylformamide (11).

Our study suggests that there is little difference in the final result if biotin or digoxigenin is used as the DNA-labeling moiety. Likewise, all three detection reagents, exhibited similar detectabilities but the speed of detection is highest with AMPPD, followed by HNPP and BCIP/NBT. During our initial optimization experiments, we found that the most profound effect on the final result was caused by the washing protocols after hybridization. Incomplete washing resulted in high backgrounds while excessive washing lead to deterioration of sensitivity due to probe removal from the hybrid.

In conclusion, we have demonstrated that single copy genes can be detected on Southern blots from as little as 0.3 μg of total genomic DNA by using either biotin or digoxigenin as labels and either AMPPD, BCIP/NBT or HNPP as substrates of alkaline phosphatase-based detection. Either of these labeling and detection methods could form the basis of highly sensitive nonisotopic nucleic acid hybridization systems that could find routine application in the clinical laboratory.

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