Volume 13 Number 22 1985 Nucleic Acids Research

Detection of subpicogram quantities of specific DNA sequences on blot hybridization with biotinylated probes

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Received 30 July 1985; Revised and Accepted 28 October 1985

ABSTRACT

A sensitive method for detecting biotinylated DNA probes on dot and Southern blots is described which is based on the principle outlined by Leary et al (1). This system has two main components: detection of biotinylated DNA by a two-step procedure with streptavidin and poly(alkaline phosphatase); and blocking background with Tween 20. 32fg and 80fg of λ phage DNA was detected on dot and Southern blot hybridizations respectively. 150fg of β-globin was detected on Southern blots of genomic DNA. This method is fast, reproducible and can detect single copy genes in 0.25μg of human genomic DNA on Southern blots.

INTRODUCTION

Molecular hybridization is used for detecting specific nucleic acid sequences in experimental and clinically derived samples. In matrix hybridizations the probe is usually labelled with radioactive phosphate (^{32}P). However, safety, disposal, short half-life, and duration of autoradiographic exposure limit the usefulness of ^{32}P-probes in clinical laboratories. Radioactive thio-labelled DNA probes overcome some of the problems associated with ^{32}P (2). Another approach has been to develop detection systems for probes labelled with a non-radioactive reporter molecule. The reporter molecule may be a hapten (3-5), protein (6-10) or biotin (1,11-19) and these have been detected by various methods (1,20,21). Of these, biotin is usually preferred since it minimally interferes with hybridization efficiency and can be detected by sensitive and specific procedures (1,20).

With current nonisotopic probing methods, the lower limit of sensitivity is of the order of pg on blot hybridizations (1,5,8-10,19). Here, a procedure is described which detects about 100fg of DNA on dot and Southern blot hybridization with biotinylated probes. The procedure is rapid and reproducible on human genomic DNA.

MATERIALS AND METHODS

λ phage and Hind III digested λ phage DNA were obtained from Boehringer Mannheim. Bovine serum albumin (BSA), polyvinylpyrrolidone (PVP 360), sodium dodecyl sulfate (SDS), calf thymus DNA, herring sperm DNA, agarose, Tween 20, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and thymidine 5'-triphosphate (TTP) were the products of Sigma (U.K.). Restriction enzymes were purchased from Bio-Labs (U.K.); dextran sulfate 500 and Ficoll 400 from Pharmacia; dimethylformamide from Aldrich (U.K.); nick translation kits from Enzo (U.S.A.) and Bethesda Research Laboratories (BRL); biotinylated poly(alkaline phosphatase) from BRL. Nitrocellulose filters were obtained from Schleicher and Schuell (Germany). Streptavidin was donated by Amersham International (U.K.).

Methods

Dot blots. Biotinylated λ phage DNA, and Hind III digested λ phage DNA, were serially diluted in carrier DNA (2.5mg/ml) in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). The diluted DNA was heat-denatured in boiling water for 10 min, cooled immediately on ice, and spun briefly. 2µl of dilutions were spotted onto nitrocellulose pre-equilibrated with 20 x SSC for 10 min at 22°C; 1 x SSC = 150mM NaCl, 15mM trisodium citrate, pH 7.0 (22). Filters were washed briefly with 20 x SSC, followed by 6 x SSC for 5 min at 22°C. Filters were blotted dry and baked at 80°C for 2 h.

Southern blots. Hind III digested λ phage DNA was diluted with sheared herring sperm DNA (1mg/ml) in TE buffer and heated at 55°C for 10 min. The digested fragments were fractionated by agarose gel electrophoresis (0.7%, w/v, agarose in 67mM Tris, 67mM boric acid, 1.5mM EDTA, pH 8.0). Normal human DNA was digested with restriction enzymes. Restriction fragments were fractionated by agarose gel electrophoresis. After electrophoresis, the gels were washed briefly with water, immersed in 0.25M HCl for 20-30 min at 22°C (23), followed by 2 x 15 min in denaturing buffer (1M NaCl, 0.5M NaOH) then 2 x 15 min in neutralising buffer (3M NaCl, 0.5M Tris-HCl, pH 7.4). Denatured DNA fragments were transferred from agarose to nitrocellulose by capillary flow of 20 x SSC (24) as modified (22). After transfer, filters were washed with 6 x SSC at 22°C for 5 min, blotted dry and baked at 80°C for 2 h.

Biotinylation of DNA probes. Double stranded DNA probes were labelled with biotin by nick translation; the manufacturer's procedure was used with minor modification . In some cases, TTP was added to give a final concentration of 2-4µM. The reaction mixture was incubated at 15°C for 90-120 min. For

optimal hybridization signal, the percent substitution and average fragment length (after denaturation) of the probes was controlled at approximately 30% (30% of thymidyl residues were substituted by biotinylated deoxyuridyl residues) and 0.6Kb respectively.

Hybridization. Filters were immersed in water until they were evenly wet and placed in plastic bags. Prehybridization cocktail (10ml/100cm^2 filter) containing 4xSSPE (1xSSPE=180mM NaCl, 10mM sodium phosphate, 1mM EDTA, pH7.4) 6 x Denhardt's solution (25) (1 x Denhardt's solution=0.02% BSA, PVP 360, and Ficoll 400, w/v), 300µg/ml sheared herring sperm DNA, 0.1% (w/v) SDS, was added to the bag without introducing air bubbles. The bags were sealed and filters incubated at 65°C for 4-6 h. After prehybridization, a corner of the bag was cut and prehybridization cocktail squeezed out. Hybridization cocktail (4ml/100cm^2 filter) containing 4 x SSPE, 2 x Denhardt's solution, 200µg/ml sheared herring sperm DNA, 0.1% SDS, 10% (w/v) dextran sulfate (23), and 20-75ng/ml biotinylated probe (denatured by boiling) was added to the bag without introducing air bubbles. The bags were resealed and the filters hybridized at 65°C for 16-20h. After hybridization, the filters were briefly rinsed in 2 x SSC containing 0.1% SDS and washed with 0.1% SDS in 2 x SSC for 3 x 5 min at 22°C, followed by 0.1% SDS in 0.25 x SSC for 3 x 5 min at 22°C. The filters were finally washed at 55°C with 0.1% SDS in 0.25 x SSC for 3 x 15 min.

Detection of biotinylated probes. Filters were washed with blocking buffer (100mM NaCl, 100mM Tris-HCl, 3mM $MgCl_2$, 0.5% Tween 20, v/v, pH 7.5) at 22°C for 90 min with vigorous shaking. Streptavidin (2µg/ml) in incubation buffer was added to the filter at 22°C for 10 min with gentle rocking; the incubation buffer was similar to the blocking buffer except that it contained 0.05% Tween 20. Filters were washed with shaking, at 22°C for 3 x 5 min with blocking buffer. Biotinylated poly(alkaline phosphatase), 1µg/ml in incubation buffer, was added to filters and incubated as for streptavidin; the volume of streptavidin and biotinylated poly(alkaline phosphatase) was 3-4ml/100cm^2 filter. After incubation, filters were washed in blocking buffer for 3 x 5 min at 22°C with shaking, then washed with developing buffer (100mM NaCl, 100mM Tris-HCl, 10mM $MgCl_2$, pH 9.6) for 2 x 5 min at 22°C.

Fresh stock solutions of NBT (75mg/ml) in 70% dimethylformamide (v/v), and BCIP (50mg/ml) in 100% dimethylformamide were used as substrates for alkaline phosphatase detection. For use, the substrate solution was made by adding 4.4µl stock NBT and 3.3µl stock BCIP to 1ml of developing buffer

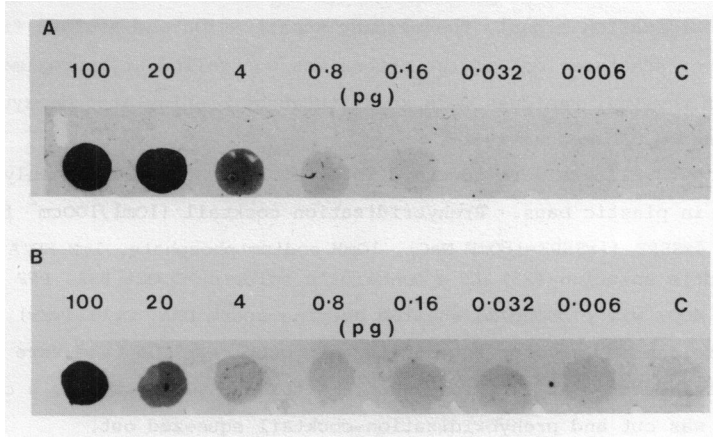

FIGURE 1. (a) Detection of biotinylated λ phage DNA. The amount of biotinylated DNA in each dot is 100pg to 6.5fg in 5-fold dilutions. 5µg of calf thymus DNA was used as carrier DNA on each dot; the last dot is control (c) carrier DNA only.
(b) Dot blot hybridization of Hind III digested λ phage DNA. Each dot is as for Figure 1a except that 5µg of sheared herring sperm DNA was used as carrier. Hybridization was performed at a probe concentration of 75ng/ml for 17h at 65°C. The colorimetric reaction is non linear over 16h with alkaline phosphatase substrate.

to achieve a final concentration of NBT and BCIP of 330µg/ml and 167µg/ml respectively. Filters were incubated with substrate solution for 16-20 h at 22°C in plastic petri dishes. After colour development, the filter was extensively washed with water and stored dry in plastic bags.

RESULTS

Figure 1 shows a typical dot titration of biotinylated λ DNA, (Fig. 1a) and λ DNA probed with biotinylated λ phage DNA (Fig. 1b). A positive signal at 32fg can be clearly seen (Fig. 1a). On this black and white figure there appears to be a signal on the control dot. This is apparent rather than real because the control dot is yellow in colour and the λ phage positive signal is purple; the yellow colour may be due to the precipitation of NBT. On dot blot hybridization 32fg could also be detected (Fig. 1b). A purple colour reaction does develop with carrier DNA even with stringency washes at 60°C. This appears to be non-specific binding of probe to carrier since it is not observed when probe is omitted from the hybridization mix. This problem with carrier DNA can be overcome by reducing the time of incubation with substrate solution, or scanning the

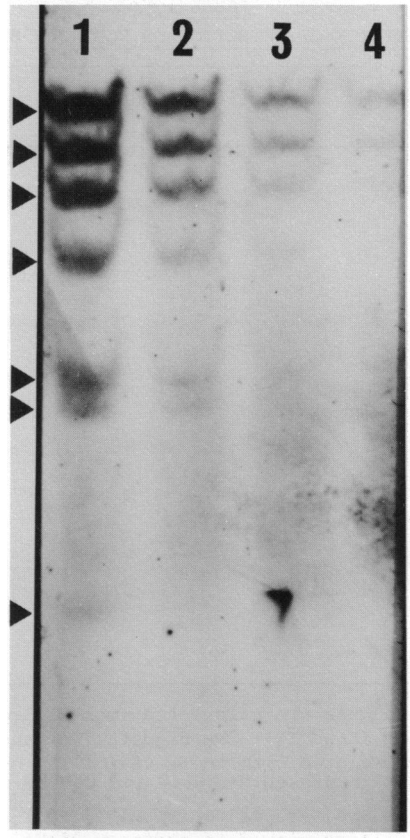

FIGURE 2. Southern blot hybridization of Hind III λ phage DNA. The total amount of λ DNA on each lane is 20pg (lane 1), 6.6 (lane 2), 2.2 (lane 3) and 0.74pg (lane 4). 5µg of sheared herring sperm carrier DNA was present in each lane. The size of each fragment is indicated in Table 1. Hybridization was performed at a probe concentration of 75ng/ml for 17h at 65°C.

filter densitometrically and subtracting the base line value from specific signal. The simplest way to control the signal to noise ratio is to observe the filter by transillumination. In this way, non-specific spots can be eliminated and only the specific signals seen.

Figure 2 shows a typical Southern blot of Hind III digested λ phage DNA. The 7 restriction fragments are easily seen in lanes 1 and 2. The size, DNA mass in each band, and its detectability, (Fig. 2, lanes 2 and 4) are listed in Table 1. From the latter it is evident that about 80fg of λ DNA can be visualised.

TABLE 1

SENSITIVITY OF DETECTION ON SOUTHERN BLOTS

DNA Size (Kb)	Quantity of DNA[1] (pg)	SIGNAL[2]
23.61	3.20	+ve
	0.36	+ve
9.64	1.31	+ve
	0.15	+ve
6.64	0.90	+ve
	0.10	+ve
4.33	0.59	+ve
	0.07	+/-ve
2.26	0.31	+ve
	0.03	-ve
1.99	0.27	+ve
	0.03	-ve
0.56	0.08	+ve
	0.01	-ve

[1] These representative data are calculated from Figure 2 (lanes 2 and 4). The fragment size of (Hind III) DNA digests (Kb) and their corresponding quantity (pg) is correlated with their visibility on filters. Bands 5 and 6 in lane 3(Fig.2) representing 0.10 and 0.09pg respectively are also visible.

[2] Signal was scored as +ve, -ve or equivocal (+/-ve) depending on its visibility on wet filters viewed in ordinary light.

Figure 3 (a and b) shows typical Southern blots of human genomic DNA probed with biotinylated c-Ha-ras and β-globin respectively. Sequences complementary to the probes can be picked up when 0.25μg of total human DNA was used. 0.5pg of c-Ha-ras and 0.15pg of β-globin were easily detected in whole human genomic DNA.

DISCUSSION

The development of nonisotopic methods for specific nucleic acid sequence detection on solid matrices has been stimulated by the inherent disadvantages of radiolabelled probes. Nonisotopic detection systems however, have normally lacked sufficient sensitivity for routine use. The procedure presented here has increased the sensitivity of specific DNA detection with biotinylated probes to a level approximate to that achievable

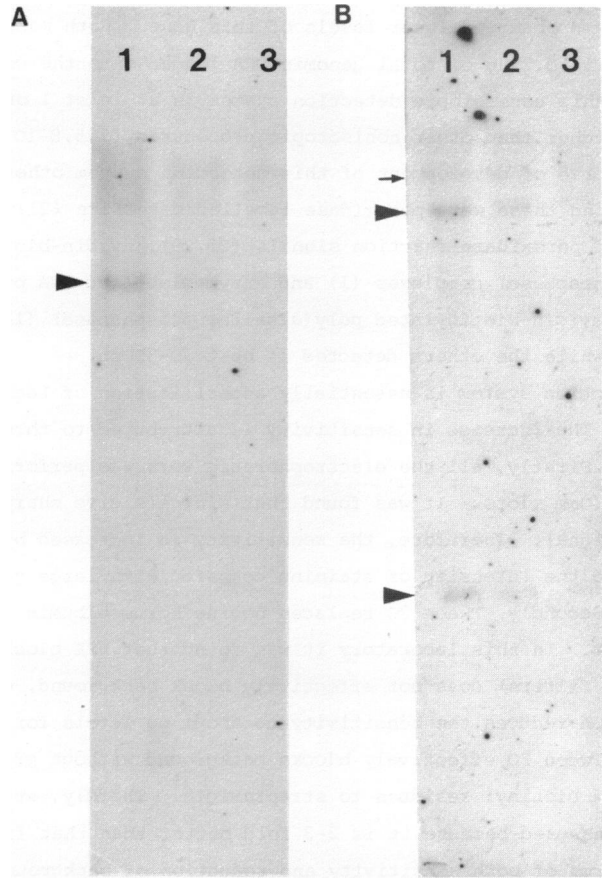

FIGURE 3. Southern blot hybridization of human DNA. Normal human DNA (from 1 patient) was digested with Bam HI and fractionated by 0.7% agarose gel electrophoresis. The amount of digested DNA in lanes 1-3 was 1, 0.5 and 0.25µg diluted in TE buffer. The filters were hybridized with a) 60ng/ml biotinylated c-Ha-ras (pEJ6.6) (26) for 17h at 65°C. The c-Ha-ras 6.8kb fragment is indicated; there is weak cross hybridization with larger fragments. b) 20ng/ml biotinylated β-globin (Pst β 4.4) (27) for 17h at 65°C. The 8.3 and 1.8kb fragments of β-globin are indicated. This probe cross hybridizes with δ-globin and gives a weak signal with only the 13.5kb fragment.

with radiolabelled probes. About 30fg of λ phage DNA was visualised on dots and dot blot hybridizations; 80fg of λ phage DNA on Southern blots; and 150fg of β-globin on genomic Southern blots. In the experiments shown 550fg of c-Ha-ras were also detected on Southern blots. Because of the

intensity of the band obtained with biotinylated c-Ha-ras it is probable that we could have observed lower levels of this gene. Both mammalian genes were visualised in 0.25µg of total genomic DNA loaded onto the gels. The sensitivity of this nonisotopic detection system is at least 1 order of magnitude higher than other nonisotopic procedures (1,5,8-10,19).

In the course of development of this detection system other methods were tried. Among these were peroxidase labelled detectors (21), amplification of peroxidase reaction signals (28,29), avidin-biotinylated poly(alkaline phosphase) complexes (1) and enzyme labelled DNA probes (9). Of these, only avidin-biotinylated poly(alkaline phosphatase) (1) detected less than 10pg while the others detected at best 10-500pg.

This detection system is essentially a modification of Leary et al's (1) procedure. The increase in sensitivity is attributed to three major modifications. Firstly, all the electrophoretic work was performed on minigels with 3.0mm slots. It was found that minigels give sharper bands of hybridization signal. Therefore, the sensitivity is increased by 3-4 fold by concentrating the intensity of staining compared with large gels having bigger slots. Secondly, Tween 20 replaces bovine serum albumin (BSA) as blocking reagent. In this laboratory it was found that BSA blocking (without baking filters) does not effectively block background, while baking filters after BSA reduces the sensitivity to about pg levels for λ phage DNA on dot blots. Tween 20 effectively blocks background without affecting accessibility of biotinyl residues to streptavidin. Thirdly, streptavidin from Amersham was used because it is 2-3 fold better than that from other suppliers in terms of both sensitivity and reduction of background and non-specific binding; the ratio of signal to noise is thus increased by at least 4-9 fold. It should be emphasised that in order to retain the sensitivity, oxidation during baking or on storage of nitrocellulose must be avoided by storing filters under vacuum. The two-step system for detection of biotin, streptavidin followed by poly(alkaline phosphatase), gives the same sensitivity as the single step procedure (1) but background is reduced. Filters can be incubated, therefore, in substrate for longer (48-72h) without significant increase in background noise provided substrate solution is changed daily. The simplicity, sensitivity and speed of the procedure will enable single copy gene analysis to be performed on extracts of small clinical biopsy samples. Therefore, investigation of the molecular pathology of disease processes in solid tissue samples will be facilitated.

ACKNOWLEDGEMENTS

V. T-W. C was a T.C. Postgraduate Scholar, and is partially supported by an O.R.S. award. The work was funded by grants from the Cancer Research Campaign (U.K.).

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