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Practical and Economical Method for Using Biotinylated DNA Probes with Bacterial Colony Blots To Identify Diarrhea-Causing *Escherichia coli*

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A simple and economical method was developed for using biotinylated DNA probes to hybridize with bacterial colonies belonging to the various categories of diarrhea-causing *Escherichia coli*. Simplification and cost containment were achieved by using Whatman no. 541 filter papers instead of nitrocellulose, by minimizing the concentration of proteinase K (an expensive but necessary reagent used to pretreat the colony blots prior to hybridization with biotin-labeled DNA probes) and by reusing hybridization solution containing labeled probe DNA. After exposing the colony blots to lysing solution and steam, followed by lysozyme (1.5 mg/ml), sucrose (25%), and proteinase K (10 μg/ml) treatments, biotinylated probes were used to detect enterotoxigenic, enteropathogenic, enterohemorrhagic, diffuse adherence, and enteroinvasive categories of diarrhea-causing *E. coli* with a high level of sensitivity and specificity. Three independent observers who were experienced in reading DNA blots recorded remarkably similar results, while less satisfactory results were obtained when the blots were read by an inexperienced observer. This technique will be useful in laboratories in which radioactive isotopes are unavailable or impractical and in which budgets are restricted.

Prior to the availability of DNA probes, the epidemiology of diarrhea-causing *Escherichia coli* was studied by using immunoassays and bioassays to phenotypically identify virulence factors, such as toxins. This was cumbersome and expensive. The development of DNA probes that detect isolates belonging to the major categories of diarrhea-causing *E. coli* with a high degree of sensitivity and specificity was an important advance, enabling epidemiological studies to be supported by a single assay (2, 6, 14, 19, 25, 26). However, perhaps with the exception of enterohemorrhagic *E. coli* (EHEC) (13, 15, 17), the other categories of diarrhea-causing *E. coli*, including enterotoxigenic *E. coli* (ETEC) (2, 6, 13, 14, 26), enteroinvasive *E. coli* (EIEC) (2, 6, 13, 14, 25, 26), enteropathogenic *E. coli* (EPEC) (2, 6, 13, 14, 20, 26), diffuse adherence *E. coli* (DAEC) (14), and enteroaggregative *E. coli* (1–4), cause disease primarily in less-developed countries.

Early methods incorporated [α-^{32}P]dATP into the DNA probes as a marker and used nitrocellulose or Whatman no. 541 paper filters as a solid support (8, 15, 16, 19, 20, 22, 24, 27). [α-^{32}P]dATP can now be replaced with biotin-dATP (7, 9, 10, 11, 21). Using Whatman no. 541 paper filters and biotin-dATP would make it feasible to use this technique for large-scale epidemiological studies in laboratories with limited budgets. The biotinylated probe method exploits the high affinity of streptavidin for biotin-labeled molecules in a sandwich system analogous to an indirect enzyme-linked immunosorbent assay. A streptavidin-alkaline phosphatase conjugate is used to colorimetrically detect biotin-labeled DNA probes which have hybridized to target DNA present on the filter paper.

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Herein we describe a simple, economical method for using biotin-labeled DNA probes that is amenable to screening large numbers of *E. coli* colony blots. The technique was designed for an epidemiological field study of *E. coli* diarrhea in two cohorts of children under long-term surveillance in Santiago, Chile.

MATERIALS AND METHODS

***E. coli* strains.** A series of well-characterized ETEC, EPEC, EHEC, EIEC, and DAEC strains from the Center for Vaccine Development collection were used in this study.

Colony blots. The *E. coli* strains were inoculated onto MacConkey or Luria agar plates (40 colonies per plate, plus a positive and negative control) in a grid pattern. After overnight growth at 37°C, a Whatman no. 541 filter paper was pressed evenly over the surface of the plate and removed, lifting the colonies with the filter paper. Filters were placed with the colony side up in a petri dish containing a Whatman no. 3 filter paper saturated with a 0.5 M NaOH–1.5 M NaCl solution and steamed in an autoclave (with the door closed but not sealed) for 10 min to lyse bacteria and denature DNA (16). The Whatman no. 541 filters were placed upon another Whatman no. 3 filter paper saturated with 1.0 M Tris hydrochloride, pH 7.0, and 2.0 M NaCl for 10 min to neutralize the NaOH. The filters were air dried and either used immediately or saved.

Treatment of filters to remove colony debris. The effectiveness of lysozyme-sucrose and proteinase K in removing bacterial debris was assessed by exposing colony blots to lysozyme-sucrose alone, proteinase K alone, a combination of the two, or neither. A modification of the method of Haas and Fleming was used (9).

For treatment with lysozyme-sucrose alone, filters were rinsed three times in cold (4°C) 0.05 M Tris hydrochloride,

TABLE 1. Effect of lysozyme-sucrose and proteinase K on hybridization

Treatment[a]	Observer[b]	Result with DNA probe[c]: EAF Sens	EAF Spec	LT Sens	LT Spec	EIEC Sens	EIEC Spec	EHEC Sens	EHEC Spec	DAEC Sens	DAEC Spec
None	1	UR	UR	90	100	UR	UR	100	100	UR	UR
	2	UR	UR	100	100	UR	UR	100	100	UR	UR
	3	UR	UR	100	100	UR	UR	100	100	UR	UR
	4	UR	UR	90	100	UR	UR	90	100	UR	UR
LS	1	100	100	100	100	95	50	100	100	100	80
	2	100	100	100	100	80	85	100	100	95	95
	3	100	100	100	100	85	100	100	100	100	90
	4	100	100	100	100	85	60	100	100	100	90
PK	1	95	95	100	100	75	55	100	100	90	95
	2	95	100	100	100	50	65	100	100	80	100
	3	95	100	100	100	35	80	100	100	80	100
	4	95	100	100	100	70	35	100	100	UR	UR
LS + PK	1	100	100	100	100	85	90	100	100	85	90
	2	100	100	100	100	85	95	100	100	85	95
	3	100	100	100	100	85	100	100	100	85	90
	4	100	75	100	100	85	85	100	100	85	85

[a] LS, Lysozyme (1.5 mg/ml) and 25% sucrose; PK, proteinase K (100 μg/ml).
[b] Observers 1, 2, and 3 were experienced with reading DNA colony blots, and observer 4 was experienced with reading immunoblots, but not colony blots.
[c] Sens, Sensitivity; defined as the number of true positives correctly identified/20 (total number of true positives); Spec, specificity; defined as the number of true negatives correctly identified/20 (total number of true negatives); UR, unreadable.

pH 8.0; placed in 1.5 mg of lysozyme (L 6876; Sigma Chemical Co., St. Louis, Mo.)–25% sucrose per ml of cold (4°C) 0.05 M Tris hydrochloride (pH 8.0) (2 to 3 ml per filter) for 10 min; and then rinsed with vigorous agitation in prewarmed (42°C) SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.2) (9) (step 1).

For treatment with proteinase K alone, filters were rinsed in prewarmed (42°C) SSC and incubated in 100 μg of proteinase K (P-0390; Sigma) per ml of SSC (2 to 3 ml per filter) for 1 h at 42°C (9) (step 2).

These two steps were combined for filters treated with both lysozyme-sucrose and proteinase K. All filters were subsequently rinsed with SSC at ambient temperature and air dried (step 3).

Evaluation of different concentrations of proteinase K. Filters were treated as described in steps 1 and 2. The proteinase K concentrations used included 10, 100, and 1,000 μg/ml. To assess the reproducibility of the technique, additional experiments using 10 or 100 μg of proteinase K per ml were performed by using three of the five probes.

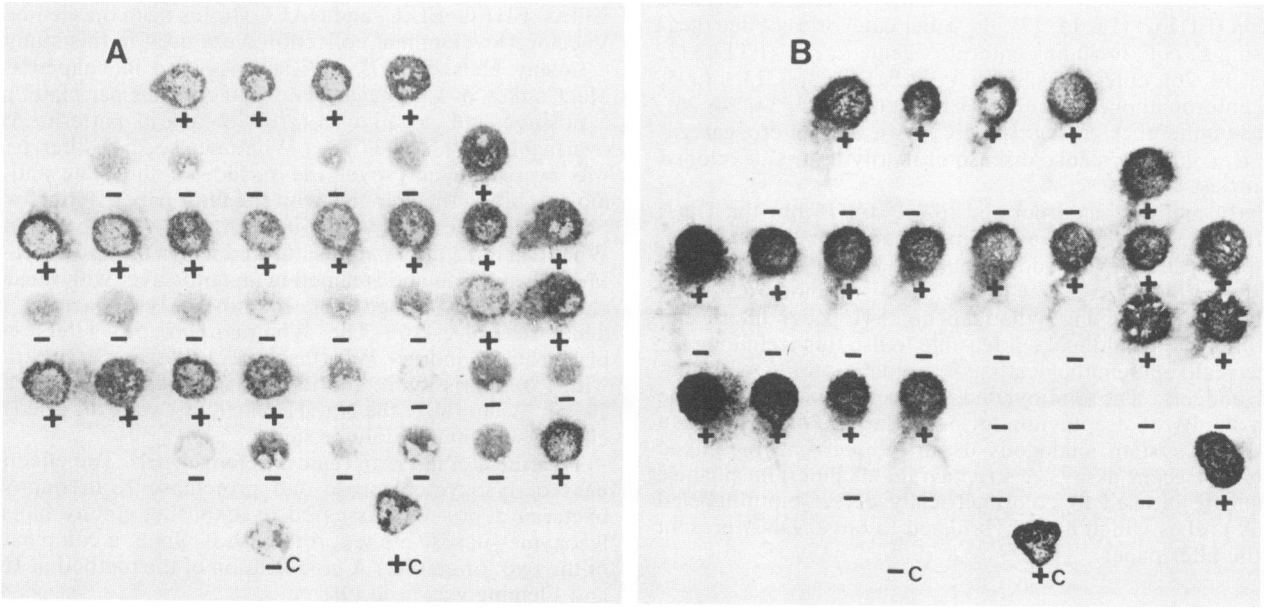

FIG. 1. Whatman filter paper containing EHEC and other *E. coli* colonies hybridized with the EHEC DNA probe. (A) Filter not pretreated with lysozyme-sucrose or proteinase K; (B) filter pretreated with lysozyme-sucrose followed by proteinase K.

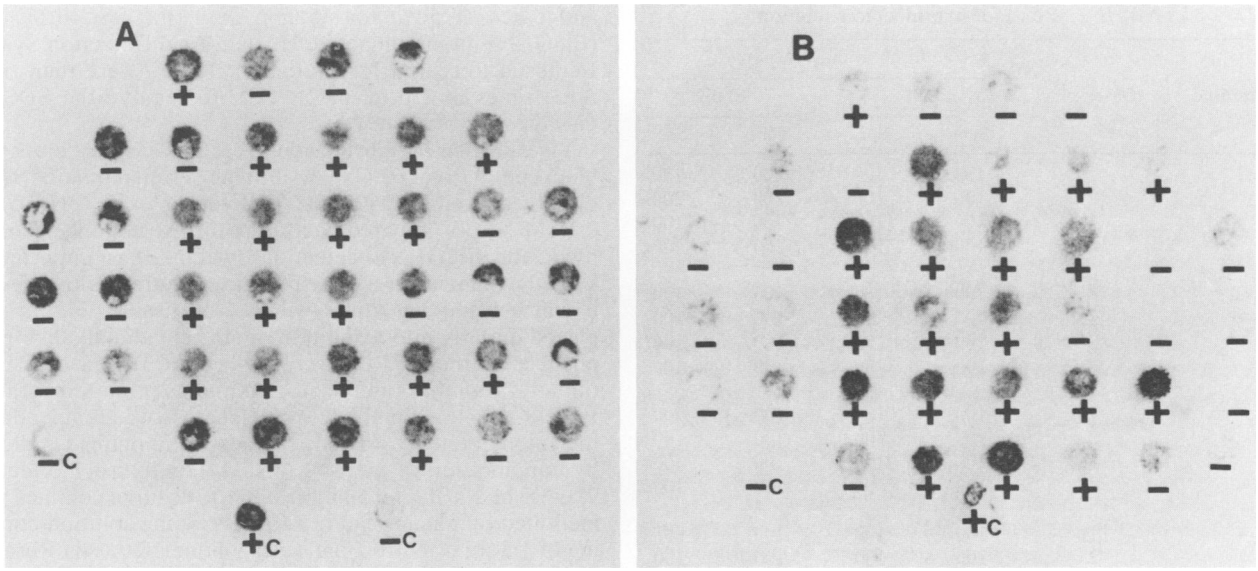

FIG. 2. Whatman filter paper containing EIEC and other *E. coli* colonies hybridized with the EIEC DNA probe. (A) Filter not pretreated with lysozyme-sucrose or proteinase K; (B) filter pretreated with lysozyme-sucrose followed by proteinase K.

TABLE 2. Effect of proteinase K concentration on hybridization

Treatment[a]	Observer[b]	Result with DNA probe[c]: EAF Sens	EAF Spec	LT Sens	LT Spec	EIEC Sens	EIEC Spec	EHEC Sens	EHEC Spec	DAEC Sens	DAEC Spec
LS + 10 μg of proteinase K per ml											
Experiment 1	1	100	95	100	100	90	90	100	95	85	100
	2	100	95	100	100	90	85	100	95	85	100
	3	100	100	100	100	80	100	100	95	85	100
	4	100	95	100	100	95	85	100	95	90	100
Experiment 2	1	95	100	100	100	60	95	ND	ND	ND	ND
	2	95	95	100	100	70	95	ND	ND	ND	ND
	3	100	100	100	100	75	80	ND	ND	ND	ND
	4	100	50	100	35	75	80	ND	ND	ND	ND
LS + 100 μg of proteinase K per ml											
Experiment 1	1	100	90	100	100	85	95	100	100	85	100
	2	100	95	100	100	85	90	100	100	85	100
	3	100	100	100	100	80	100	100	100	85	100
	4	100	95	100	100	85	95	100	100	85	95
Experiment 2	1	80	100	100	100	70	95	ND	ND	ND	ND
	2	90	95	100	100	70	90	ND	ND	ND	ND
	3	95	100	100	100	70	90	ND	ND	ND	ND
	4	100	85	100	45	75	45	ND	ND	ND	ND
LS + 1 mg of proteinase K per ml	1	100	90	100	100	95	80	100	95	100	90
	2	100	100	50	100	90	85	100	100	100	90
	3	100	100	100	100	80	100	100	100	100	90
	4	100	95	95	100	85	90	100	100	100	90

[a] LS, Lysozyme (1.5 mg/ml) and 25% sucrose.
[b] Observers 1, 2, and 3 were experienced with reading DNA colony blots, and observer 4 was experienced with reading immunoblots, but not DNA colony blots.
[c] Sens, Sensitivity; defined as the number of true positives correctly identified/20 (total number of true positives); Spec, specificity; defined as the number of true negatives correctly identified/20 (total number of true negatives); ND, not done.

TABLE 3. Reuse of hybridization solution

Times used	Observer[a]	Result with DNA probe[b]: EAF Sens	EAF Spec	EHEC Sens	EHEC Spec
1	1	100	100	100	100
	2	100	100	100	100
	3	100	100	100	100
	4	100	100	100	100
2	1	100	100	100	100
	2	100	95	100	100
	3	100	95	100	100
	4	100	80	100	100
3	1	100	100	100	100
	2	100	100	100	100
	3	100	100	100	100
	4	100	90	100	100
4	1	100	100	100	95
	2	100	100	100	100
	3	100	100	100	100
	4	100	95	100	95

[a] Observers 1, 2, and 3 were experienced with reading DNA colony blots, and observer 4 was experienced with reading immunoblots, but not DNA colony blots.

[b] Sens, Sensitivity; defined as the number of true positives correctly identified/20 (total number of true positives); Spec, specificity; defined as the number of true negatives correctly identified/20 (total number of true negatives).

Preparation of biotinylated probes. DNA probes to detect EHEC, EPEC, EIEC, heat-labile enterotoxin (LT)-producing ETEC, and DAEC were prepared. The LT probe is a 1-kilobase *Bam*HI fragment derived from pWD299 (12, 19, 23). Briefly, the *Hin*cII fragment was removed from pWD299, *Bam*HI linkers were attached, and this fragment was cloned into pACYC184 and called pCVD403. The EHEC DNA probe is a 3.4-kilobase *Hin*dIII fragment from pCVD419 (15). The EPEC adherence factor (EAF) probe for detecting EPEC is a 1-kilobase *Sal*I-*Bam*HI fragment derived from strain E2348/69 (20). The EIEC probe is a 2.5-kilobase *Hin*dIII fragment of pSF55 derived from the epithelial cell invasiveness plasmid (plnv) of the EIEC strain *E. coli* 11 (22, 27). The DAEC probe is a 390-base-pair *Pst*I fragment from pSLM862 cloned into pUC8 (5).

The DNA probes were labeled with biotin-7-dATP (Bethesda Research Laboratories, Gaithersburg, Md.) by nick translation (18). Separation of unincorporated biotin-7-dATP from labeled probe DNA was not necessary. Immediately prior to use, probes were denatured to single strands of DNA by boiling for 10 min and then chilled on ice to prevent renaturation.

Hybridization. The processed filters were hybridized overnight at 42°C by a modification of the method of Sethabutr et al. (21) in a hybridization solution containing 45% formamide, 4× Denhardt solution (1× Denhart solution is 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 4× SET buffer (1× SET buffer is 0.15 M NaCl, 0.03 M Tris hydrochloride [pH 8.0], and 1 mM EDTA), 0.5% sodium dodecyl sulfate (SDS), 24 μg of heat-denatured salmon sperm DNA per ml, and 40 ng of probe DNA per ml (2 ml of hybridization solution per filter).

After overnight hybridization, the filters were washed twice for 3 min in room temperature 2× SSC–0.1% SDS, twice for 3 min in room temperature 0.2× SSC–0.1% SDS, and twice at 50°C for 15 min in 0.16× SSC–0.1% SDS (BluGENE Nonradioactive Nucleic Acid Detection System; Bethesda Research Laboratories). Filters were then rinsed three times in 250 ml of 2× SSC to remove the SDS and visualized immediately.

Visualization of hybridization reaction. Colony blots were blocked for 1 h at 62°C in 3% bovine serum albumin (Sigma) dissolved in buffer 1 (0.1 M Tris hydrochloride (pH 7.5) and 0.15 M NaCl) prepared according to instructions accompanying the BluGENE detection system. It is important to keep the temperature of the blocking solution below 65°C, or it will gel and the filters will not be usable. Filters were placed directly into a solution of streptavidin-alkaline phosphatase (Bethesda Research Laboratories) at a concentration of 1 μg/ml in buffer 1 for 10 min at room temperature. Excess streptavidin-alkaline phosphatase was removed from the colony blots by washing them twice in buffer 1, followed by equilibration in buffer 2 (0.1 M Tris hydrochloride, pH 9.5; 0.1 M NaCl; and 50 mM $MgCl_2$). Positive colonies were identified by placing filters in a developing solution consisting of 1.5 μg of Nitro Blue Tetrazolium (Bethesda Research Laboratories) and 0.6 μg of 5-bromo-4-chloro-3-indolylphosphate (BRL) per ml of buffer 2. The signal from positive colonies was easily distinguishable from background in 1 to 3 h.

Blinded observers. All processed filters were coded and given to four independent observers to be read, including three observers experienced in reading biotinylated DNA probe blots and one experienced in reading immunoassay colony blots but not DNA blots.

Reuse of hybridization solution. Colony blots containing 20 EAF or EHEC out of 40 colonies were prepared as described above. The filters were identically treated with lysozyme-sucrose followed by proteinase K (100 μg/ml) as described above in steps 1 and 2. The filters were hybridized one filter at a time by using the same hybridization solution containing either the EAF or EHEC probe at 40 ng/ml in a volume of 10 ml per filter. Filters were washed and visualized as described above. Solutions containing DNA probes were stored at 4°C between hybridizations.

RESULTS

Lysozyme-sucrose versus proteinase K. The effects of pretreating filters with lysozyme-sucrose and proteinase K as a preliminary step to hybridization with the EAF, LT, EIEC, EHEC, or DAEC biotin-labeled DNA probe are shown in Table 1. Results varied markedly, depending on the particular probe. With two probes (LT and EHEC), high levels of sensitivity and specificity were obtained even without enzyme treatment (Fig. 1). However, the remaining three probes required that the filters be pretreated with at least one enzyme to obtain satisfactory results. Filters hybridized with the EIEC probe were not easily read unless they were pretreated with both lysozyme-sucrose and proteinase K. This is illustrated in Fig. 2, which represents blots that were either not pretreated (Fig. 2A) or treated with both lysozyme-sucrose and proteinase K (Fig. 2B) prior to hybridization with the EIEC probe.

Optimal proteinase K concentration. Experiments were performed to determine the lowest concentration of proteinase K that would give satisfactory sensitivity and specificity with all five DNA probes (Table 2). Following initial exposure of the filters to lysozyme-sucrose, treatment with the lowest concentration of proteinase K (10 μg/ml) gave as satisfactory results overall as treatment with higher concentrations did (Table 2).

Additional experiments were carried out to verify the reproducibility of the assay when low concentrations of proteinase K were used. After initial treatment with lysozyme-sucrose, filters were exposed to 10 or 100 μg of proteinase K per ml prior to hybridization with the LT, EAF, or EIEC DNA probe. Results are shown in Table 2 (experiment 2). As before, the experienced observers recorded satisfactory sensitivity and specificity with filters treated with lysozyme-sucrose followed by proteinase K in the lowest concentration of 10 μg/ml. The EIEC probe again gave the least satisfactory results.

Reuse of hybridization solution. A final set of experiments was undertaken to show that the hybridization solutions containing biotinylated probes can be reused (Table 3). EAF and EHEC probes were used in these experiments. At an initial probe concentration of 40 ng/ml, colony blots containing 20 EHEC colonies were sequentially hybridized three additional times with the EHEC probe, with identical results (100% sensitivity and specificity recorded by all four observers). Similar results were obtained with the EPEC probe in four sequential hybridizations of colony blot filters containing 20 EPEC strains.

DISCUSSION

Previous attempts to use biotinylated DNA probes for colony blot hybridization have given mixed results (7, 9, 10, 11, 21). We set out to develop a simple, practical, low-cost method for using biotinylated DNA probes to hybridize with bacterial colony blots, so that laboratories of moderate sophistication in less-developed countries can support epidemiologic studies. Substituting Whatman no. 541 filter papers for nitrocellulose, as originally suggested by Sethabutr et al. (21), markedly lowers costs ($0.10 for one Whatman no. 541 filter versus $1.92 for one nitrocellulose filter). Paper filters also simplify the procedure, since they are much easier to handle than nitrocellulose and do not require baking.

One potential problem with biotinylated DNA probes in screening colony blots is interpretation of the hybridization results in the face of nonspecific background color development. Treatment of the colony blots with enzymes can remove bacterial debris and improve the quality of the hybridizations. Previous published protocols used proteinase K in concentrations from 200 μg/ml to 1 mg/ml (9, 21). However, since proteinase K is quite costly, we sought to verify its usefulness. While such treatment was not required for two probes (LT and EHEC), for the remaining DNA probes tested, the use of both lysozyme-sucrose and a low concentration of proteinase K (10 μg/ml) was indeed necessary to achieve acceptable sensitivity and specificity (Tables 1 and 2).

Biotinylated DNA probes can be stored for long periods of time without the instability that occurs with radioactive probes, thereby making it theoretically possible to reuse hybridization solutions containing biotin-labeled probes (Table 3). Such reuse diminishes technician time, conserves reagents and DNA probe fragments, and simplifies the procedure by eliminating the need to prepare fresh hybridization solutions for each experiment.

An important practical lesson learned from these studies is that, not surprisingly, the prior experience of the observer in reading DNA hybridizations markedly influences the sensitivity and specificity achieved in the assay. There was extraordinary correlation among the results recorded by the three independent observers experienced in reading filters hybridized with DNA probes. In contrast, the erratic results of the observer experienced in reading immunoblots but not DNA blots underscore the need to include a period of training for any observer chosen to interpret the processed filters. We have successfully trained several visiting scientists to become proficient in interpreting results of the biotinylated probe method with 3 to 4 weeks of intensive instruction. This includes comparing results of two replicate sets of filters, one hybridized with a ^{32}P-labeled probe and the other with a biotinylated probe, under the guidance of an experienced observer.

The colony blot hybridization protocol described here is economical, gives reproducible and relatively easy-to-interpret results, and allows one to capitalize upon the inherent advantages of biotinylated DNA probes, such as their ease and safety in handling and their long shelf life. Laboratories with limited budgets and those that do not have the facilities to use radioisotopes will benefit from this technique.

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