HYBRIDIZATION OF PEROXIDASE-LABELED DNA PROBES TO MICROTITER SOLID-PHASE BOUND DNA (HYBRELISA)

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A nonradioactive DNA hybridization technique for screening large number of samples is described. The use of microtiter plates as solid-phase and of peroxidase-labeled DNA as probes, makes this technique simple, rapid, compact, and easy to perform without sophisticated equipment. The method has a sensitivity of about 5 pg of bound DNA per well, is specific and gives a linear relationship between the amount of DNA bound and the signal obtained. The buffers used have been designed for stability at room temperature and the same protocols and apparatus used for enzyme-linked immunosorbent assays can be used to analyze the results of the hybridization. The described technique should be useful in many applications.

KEY WORDS
DNA Hybridization; Elisa (HYBRELISA)

With the purpose of developing an easily scalable DNA hybridization procedure to quantify a large number of samples for diagnostic purposes, hybridization conditions were studied to use the compact 96-well microtiter plates developed for solid-phase enzyme-linked immunosorbent assays (ELISA) to get some of the advantages of these assays (Coll, 1990).

In order to bind the DNA to polystyrene wells, Protocol 1 was optimized. The denatured DNA was dried onto wells precoated with polylysine and onto wells without any precoating. The precoating step had no effect upon the hybridization result and thereafter, it was omitted. After coating, the glutaraldehyde step had to be included because its omission resulted in both high backgrounds and in signals that did not proportionally show the amount of probe hybridized. Backgrounds with absorbances of 0.5 were lowered to 0.1 or less by including a glutaraldehyde-blocking step with 1 M Glycine. Prehybridization with hybridization buffer (Sambrook et al, 1989) to the DNA-coated solid-phase, also contributed to lowering the background. Tested solid phases included, Dinatech (Plochinsen, Germany) microtiter plates of 96-well and Nunc (Kamstrup, Denmark) microtiter plates divided in rows of 8 × 2 wells (Microwell module F-16 of medium binding capacity). Results were similar in both cases. Coated solid-phases were stable for at least 2 months at 4°C when kept in closed containers with dried silica gel.

To estimate the amount of DNA that binds to the polystyrene, the DNA (pUC-18) was radioactively labeled and attached to the wells. The percentage of the DNA, which remains in the wells after the glutaraldehyde attachment and the washing, shows an optimal of 0.3% when using an input amount of DNA of 13 μg/well (Table 1). The absolute amount of DNA bound increases from 39 pg/well to 83.2 pg/well as the input DNA was increased from 13 to 130 μg/well. No more DNA could be bound to the wells by increasing to 24 hours the glutaraldehyde treatment. Sensibility of this technique could probably be increased if we could find a solid-phase with higher DNA-binding capacity. The binding varied between 5% to 29% coefficient of variation (CV) from well to well (not shown) or from 1.5% to 21.4% (CV) from experiment to experiment (Table 1).

To label the DNA probes with enzymes, a simple one-step method was chosen based on the one described for antibodies by Guedson and Avrameas (1983) and modified by Coll (1987). The optimized technique is described in Protocol 2. Free peroxidase remaining after conjugation was eliminated by chromatography over Sephadex G-200 in an attempt to further reduce the background. However, no advantage was obtained by this purification step. Because the concentration-dependent stability of peroxidase conjugates might result from the loss of heme from the peroxidase (Coll, 1987), the presence of the remaining free peroxidase in the conjugate was preferred to assure its stability. In this way, conjugates could be kept for at least 2 months at 4°C. The peroxidase activity of the conjugate remained unaltered during at least 2 days at 42°C in hybridization buffer, containing 2 μM heme. The activity disappeared from the conjugate after heating to 100°C for 5 minutes. Other methods of enzymatic labeling

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PROTOCOL 1

PREPARATION OF SOLID PHASE BOUND DNA

1. Sonicate and heat-denature the DNA to be used for coating at 1mg/mL by immersion into a boiling bath for 5 minutes and chilling on ice immediately afterwards.
2. Dilute the DNA in distilled water to 1μg/mL and add 100μl per well to the polystyrene microtiter wells of 96-well plates (Dinatech, Plochingen, Germany) or to the wells of rows of 8 × 2 wells, microwell module F-16 medium binding capacity (Nunc, Kamstrup, Denmark). Place at 42°C overnight to dryness.
3. Add 100μl per well of 1% aged glutaraldehyde and incubate 30 minutes at room temperature. Then wash with 200μl of distilled water per well.
4. Add 200μl per well of 1 M glycine, pH 5 and incubate 30 minutes at room temperature. Then, wash with 200 μl of distilled water per well.
5. Add 100μl per well of hybridization buffer (30% formamide, 6 × SSC, pH 7, 0.01% SDS, 0.2% BSA, 0.2% Ficoll, 0.2% pirrolidinpirrolidone, 10μg/mL of sonicated and denatured calf thymus DNA, and 0.01% merthiolate) and incubate at 42°C for 4 hours in a humid box.
6. Wash with 200μl of distilled water per well, dry for 2 hours at 42°C and keep at 4°C with blue silica gel in tightly closed boxes.

PROTOCOL 2

PREPARATION OF PEROXIDASE-LABELED PROBES BY THE ONE-STEP AGED GLUTARALDEHYDE METHOD

1. Sonicate and heat-denature the DNA probe at 1 mg/mL by immersion into a boiling bath for 5 minutes and chilling on ice afterwards.
2. Mix the DNA (2 μg/2 μl) with 2 mg of peroxidase (1,000 U/mg, Boehringer Mannheim, Germany) in 50μl of 10 mM sodium phosphate 0.15 M NaCl, pH 7.4.
3. Add 2μl of 25% aged-glutaraldehyde and incubate overnight at 37°C with agitation.
4. Add 150μl of 0.2 M glycine and incubate overnight at room temperature. Keep at 4°C.

PROTOCOL 3

HYBRIDIZATION TECHNIQUE

1. Dilute the DNA probes in hybridization buffer containing 2μM heme, add 100μl of the mixture per well and incubate at 42°C for 6 hours in a humid box.
2. Wash three times with 200μl per well of 10-fold diluted dilution buffer (130 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.24 mM merthiolate, 5 g/L bovine serum albumin, 0.5 g/L Tween 20, 0.2 mM phenol red, 50 mM Hepes pH 7.5).
3. Add 50 μl per well of low-background substrate buffer (150 mM sodium citrate, pH 4.8, 3 mM H_2O_2, 1 mg/mL o-phenylenediamine) and incubate at room temperature for 30 minutes. O-phenylenediamine is available in 2 mg preweighted tablets from Dakopatts (Glostrup, Dennmark).
4. Add 50μl per well of 4 M H_2SO_4 to stop the reaction.
5. Read at 492 and 620 nm. The absorbance at 620 nm is used to correct for individual nonsignificant differences between wells.

were tested by following the manufacturer's instructions, biotinilation with photobiotin acetate (Bethesda Research Lab, Maryland), and incorporation of biotin 7-dATP (Bethesda Research Lab) by either random priming or nick translation (Boehringer Mannheim, Germany).

Different hybridization buffers were tested for the hybridization. The buffer described was found to be the most suitable for the hybridization in microtiter wells (Protocols 1 and 3). Salt concentration (6 × SSC) and formamide proportion (30%) of the hybridization buffer were such that the hybridization could be carried out at 42°C (Sambroock et al, 1989). After hybridization, distilled-water washes at room temperature were enough to keep the background low. To measure the peroxidase activity, optimal conditions for ELISA were used (Coll, 1989; Martinez and Coll, 1988). The optimal time for hybridization at 42°C was 4 to 6 hours. Hybridization times of 16 to 24 hours reduced about 50% of the signal obtained and increased background. The hybridization could also be performed at room temperature

TABLE 1

ESTIMATION OF THE EFFICIENCY OF ATTACHMENT OF INPUT DNA TO THE WELLS

Pg/Well		Percentages	
Coated	Attached	Attachment	CV
130,000	83.2 ± 1.3	0.064	1.5
13,000	39.0 ± 2.8	0.3	7.1
1,300	2.8 ± 0.6	21.5	21.4

Average ± standard deviations from three different attachment reactions, each by triplicates, are given. pUC-18 was sonicated and heat-denatured before labeling by random priming, following the manufacturer's instructions. Two mg of pUC-18 were labeled by using 5 μCi of (U-^{14}C) adenosine 5'-Triphosphate, ammonium salt (525 mCi/mmol) to give a specific activity of about 1.6 × 10^6 dpm/μg. After ethanol precipitation, the labeled pUC-18 was used to coat the wells. CV = coefficient of variation.

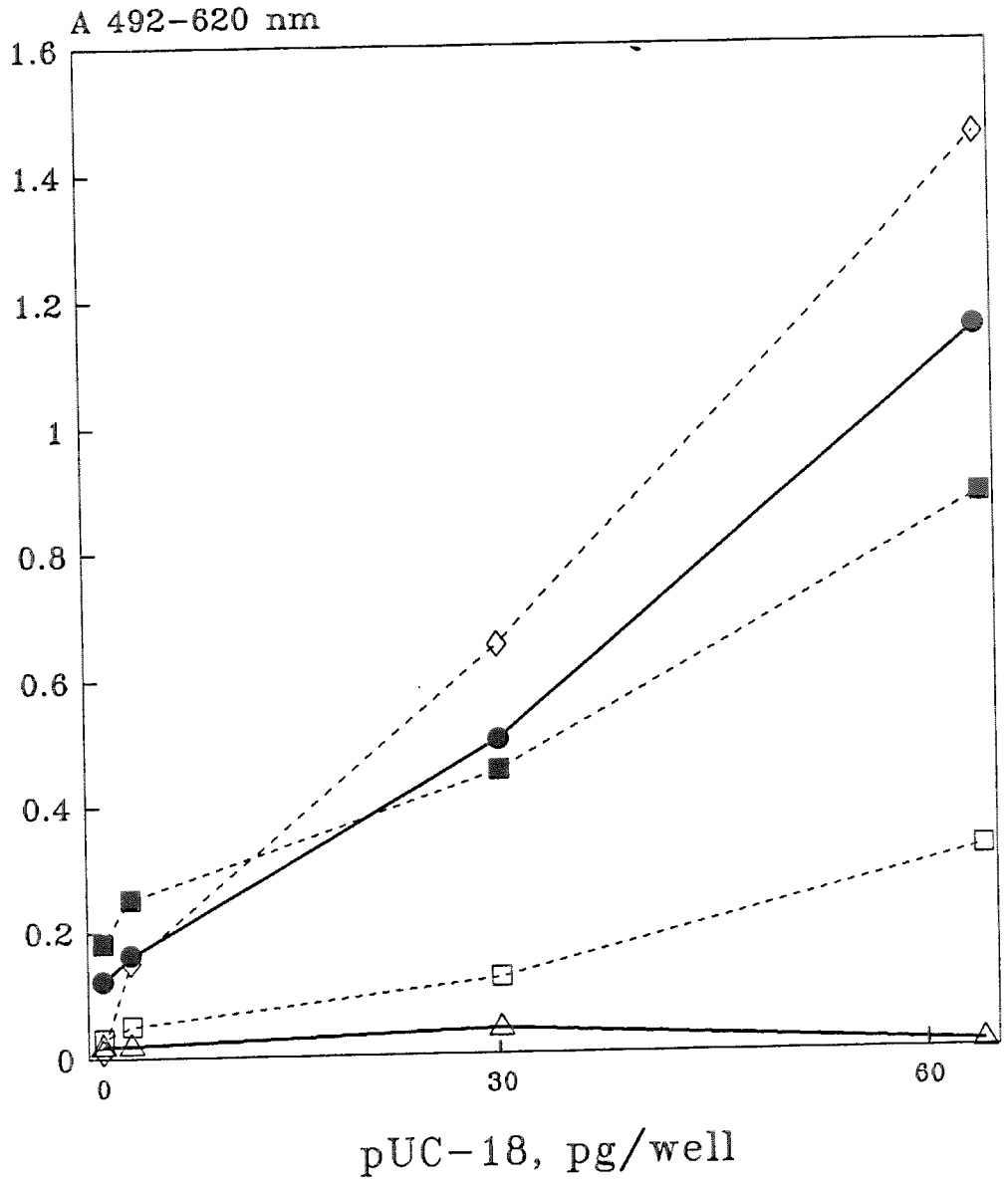

FIG 1. Comparison of different methods of labeling the DNA probes. pUC-18 at 0.12 μg/mL in water was sonicated twice, heated at 100°C and cooled down to 0°C immediately. After ethanol precipitation, the DNA was reconstituted with water to 1μg/μl, heated at 100°C and distributed in 2-μg aliquots in eppendorf tubes. Labeling reactions were made as indicated by the manufacturers and in Protocol 2 and then water was added until 200 μl total final volume. The hybrelisa was performed as indicated during 6 hours at 42°C with 10 ng of labeled probes per well. ●—●, glutaraldehyde peroxidase (Protocol 2); ■----■, photobiotin; ◇-----◇, nick translation; □----□, random priming; △—△, no pUC-18. The estimated amount of bound pUC-18 was calculated from the figures given in Table 1.

for 16 to 24 hours. The technique was optimized by using plasmid pUC-18 as a model. Figure 1 illustrates the technique's performance by comparing several methods of probe labeling. The highest signal was obtained with the DNA probe labeled with biotin-7 dATP by nick translation, but very similar results were obtained by the cheapest one-step glutaraldehyde method (Protocol 2). The specificity of the hybridization was tested by using wells coated by either undenatured double-stranded pUC-18 DNA or African Swine Fever Virus recombinant pACYC177 plasmid (Pastor and Escribano, 1990). In both cases, no signal higher than the background could be obtained (not shown).

The application of the highly stable reagents previously developed and optimized for ELISA, for example, a 2-year stable dilution buffer at room temperature (Martinez and Coll, 1988), addition of merthiolate to the hybridization buffer to increase their stability, addition of phenol red to allow visualization of the pipetted wells and to monitor the pH, possibility of using microtiter plates divided in rows that adapt the number of assays to the daily variable

number of samples, low-background substrate buffer (Coll, 1989), and use of a simple method for labeling the DNA probes, make this hybridization technique easily scalable (Coll, 1988), highly reproducible, and available for diagnostic testing of a high number of samples.

For instance, detection of viral sequences could be performed by competition to the hybridization of recombinant DNA plasmids containing the viral sequences (not shown). Polymerase chain reaction (PCR) products from virally infected samples could also be used as competitors to the hybridization in order to increase sensibility of the technique. Alternatively, PCR products can be labeled with biotin-nucleotides during the amplification reaction and directly hybridized to a solid-phase DNA plasmid carrying the viral probes (Sobrino et al, 1989).

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