

THE USE OF AMPPD AS AN ALTERNATIVE SUBSTRATE FOR AP-MEDIATED DETECTION OF NONRADIOLABELED DNA PROBES IN *Eucalyptus saligna*

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ABSTRACT

We present a non-radioactive alternative to Southern's (*J. Mol. Biol.* 98: 503-517, 1975) DNA-DNA hybridization technique. The use of AMPPD - Disodium 3-(4-Methoxyspiro [1,2-dioxetane-3,2'-tricyclo[3.3.1.1^{3,7}]decan]-4-yl)phenyl phosphate as an alternative substrate for AP-mediated detection of digoxigenin - 11 dUTP - labeled probes made possible the simple and nonhazardous reuse of blots. We used 0.8% agarose gels containing 30 µg per lane of *Eucalyptus saligna* DNA, digested with Eco RI, electrophoresed and blotted on to nylon membranes (Hybond-N, Amersham, UK), using the Southern blotting procedure, and UV irradiated for one minute for DNA fixation. The hybridizations were carried out overnight with digoxigenin labeled random inserts of *E. saligna* DNA by using the Genius Kit (Boehringer Mannheim). Detection of the DNA-DNA hybrids was performed in the presence of 0.5% blocking agent and the substrates NBT/BCIP were replaced by 0.26 mM AMPPD in the final alkaline assay buffer (50 µl/cm²). After membrane incubation for five minutes at room temperature in a sealed plastic bag, the AMPPD solution was retrieved and stored at 4°C for reuse. A Kodak X-BRAF QA-S film was pressed firmly onto the bag containing the wet membrane, exposed for two to six hours and then developed. After use, the probes were stripped off and the blots reutilized, three times so far, with the same results.

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INTRODUCTION

The genus *Eucalyptus*, nearly entirely native to Australia, has several species perfectly adapted to Brazil. For fast growth and high quality of timber and cellulose, *Eucalyptus* has proved to be the preferred genus for exploitation, having the added advantage of reducing the dependence on native species. Breeding programs, aiming to obtain hybrids for commercial purposes, use molecular techniques as an early aid to detect superior offspring.

At the moment, RFLP is being largely utilized since it gives an unlimited number of analyses, which can be studied by Mendelian procedures, and is less affected by environmental conditions (Tanksley *et al.*, 1989).

Since it is very difficult to import radioactive labeled nucleotides into Brazil, and only a few safe laboratories exist for their manipulation, the use of nonradioactive labeled DNA probes is increasing among Brazilian researchers. On the other hand, hybridization detection by colorimetric reactions makes blot reutilization difficult or even impossible. So that no advantages can be found in comparison to the conventional radioactive methods.

Allefs *et al.* (1990) suggested the use of a chemiluminescent substrate (AMPPD)* for detection of nonradiolabeled DNA probes mediated by alkaline phosphatase (AP). We have applied this technique to *Eucalyptus saligna*.

MATERIAL AND METHODS

The material utilized in the present work was kindly supplied by Bioplanta Tecnologia de Plantas Ltda, Paulínea, State of São Paulo, Brazil, from its *Eucalyptus* Germplasm Bank.

Young leaves were collected from ten individuals of *E. saligna* in nature, immediately lyophilized and stocked for future use at -18°C.

DNA was extracted according to the method of Saghai-Marooof *et al.* (1984). The introduced modifications were: adding 2% N-lauroyl sarcosine to the extraction buffer and after the first precipitation adjusting the NaCl concentration of the DNA solution to 1.5 M (Wolff and Machado, unpublished results). Thirty µg of genomic DNA were digested overnight with a 2-fold excess of Eco RI restriction enzyme, electrophoresed in a 0.8% agarose gel, and transferred to nylon membranes (Hybond-N) by using the alkaline blotting procedure of Southern (1975) as modified by Allefs *et al.* (1990).

* Disodium 3-(4-Methoxyspiro {1,2-dioxetane-3-2'-tricyclo-[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate.

Total DNA from one individual of *E. saligna* was digested with PstI and electrophoresed. Fragments with 1-2 Kb were purified by using the Genecreen kit (Bio 101), cloned into PstI-digested pEMBL plasmid vector, and transformed into *Escherichia coli* (XL1-blue) according to Chung and Miller (1988).

Randomly-selected clones used as probes, from entire linearized supercoiled plasmids were labeled with digoxigenin d-UTP, according to the Boehringer Mannheim kit instructions. Prehybridization was done at 42°C in 5X SSC 5% blocking reagent (Boehringer-Mannheim), 0.1% N-lauroylsarcosine, 0.02% SDS and 50% formamide. Overnight hybridization was carried out in the same solution, to which *E. saligna* probe had been added at a concentration of 50 ng and the blots washed in 2X SSC/0.1% SDS (2X, 20 min, RT) and 0.2X SSC/1% SDS (2X, 20 min, 65°C).

DNA-DNA hybrids were detected according to the supplier's instructions (Genius Kit, Boehringer Mannheim), with two modifications suggested by Allefs *et al.* (1990), including incubation with the antibody-AP conjugate in the presence of 0.5% blocking reagent and the replacement of the substrates NBT/BCIP by 0.26 mM AMPPD (Tropix, USA) in the final alkaline assay buffer (50 µl/cm² membrane). After incubation of the membrane for five minutes at room temperature in a sealed plastic bag, the AMPPD solution was retrieved and stored at 4°C for reuse. A Kodak X-BRAF-QA-S film was pressed firmly onto the bag containing the wet membrane, exposed for two to six hours and then developed.

The probes were then stripped off by washing twice in TE for five minutes at room temperature; twice in 0.2 M NaOH/0.1 SDS for five minutes at 37°C, twice in TE for five minutes at room temperature. After that the blots could be reused, at least three times.

RESULTS AND DISCUSSION

To date, the blots have been reutilized three times without loss of quality. Very little background was observed and this did not increase with reuse (Figure 1). Nevertheless, it was necessary to use twice as much DNA per lane to obtain the same signals on the film, when compared with the detection of radioactively labeled probes (Wolff and Machado, unpublished results). Best results were obtained by increasing blot exposure time to Kodak X-ray film, by two hours at each new reutilization.

For Brazilian conditions this methodology is suitable, since it provides means to avoid radioactively labeled nucleotide importation and does not require specially equipped laboratories. The methodology offers results of similar quality to those obtained by using radioactively labeled probes and at a lower cost.

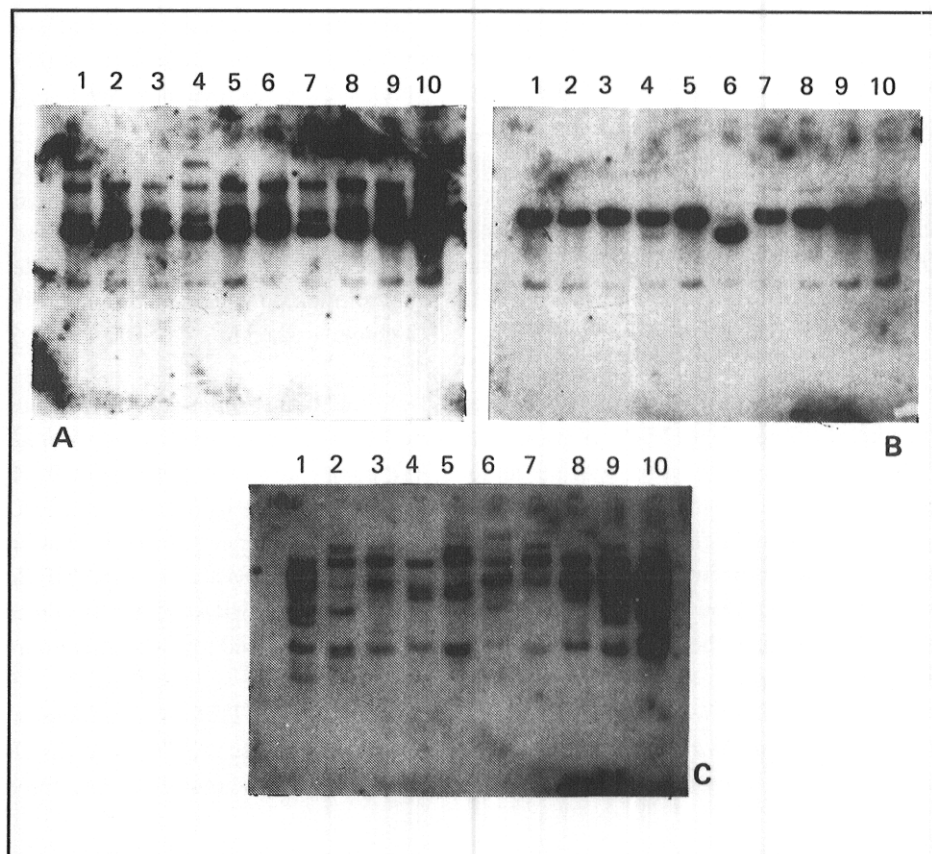


Figure 1 - Detection of *Eucalyptus saligna* single or low-copy sequences upon Southern blot hybridization using AMPPD as an alternative substrate for AP-mediated detection of nonradiolabeled DNA probes. An 0.8% agarose gel containign approximately 30 μ g aliquots of EcoRI digested total DNA of ten *E. saligna* genotypes (1 to 10) was blotted onto Hybond-N. The same membrane was blotted three times with three different randomly-selected clones from the library generated from *E. saligna* DNA. How can be seen, the same quality of results were obtained with clean blots and accurate signals in all of them (A, B, C, respectively).

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RESUMO

Desde a descrição do método original de hibridização DNA-DNA descrito por Southern (*J. Mol. Biol.* 98: 503-517, 1975), vários parâmetros têm sido modificados no sentido de otimização dessa técnica. O uso do AMPPD (Disodium 3-(4-Methoxyspiro {1,2-dioxetane-3,2'-tricyclo-[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate) como substrato alternativo para detecção de sondas marcadas não-radioativamente, permitiu a reutilização das membranas ("blots"), o que constitui grande vantagem em relação às detecções que envolvem aparecimento de cor [(Allefs *et al.* (*Nucl. Ac. Res.* 18: 3099-3100, 1990)]. Neste trabalho foram utilizados géis de agarose 0,8%, contendo alíquotas de 30 µg de DNA total de 10 genótipos de *Eucalyptus saligna* digeridos com EcoRI, transferidos alcalinamente para membrana de "nylon" (Hybond-N, Amersham, UK) e fixados com luz UV por 1 minuto. As membranas foram pré-hibridizadas por 1 hora, e hibridizadas por uma noite, com segmentos de DNA de *E. saligna* obtidos ao acaso, inseridos em plasmídeo pEMBL, clonados em XL1-Blue, marcados com digoxigenina (Genius, Boehringer Mannheim, FRG). As detecções das hibridizações foram realizadas de acordo com as especificações do "Kit" acima citado, com duas modificações: (1) incubação do anticorpo AP-conjugado em presença do "blocking agent" 0,5% e (2) substituição do substrato NBT/BCIP por 0,26 mM AMPPD, na última lavagem alcalina (5 min., à temperatura ambiente). Dentro de sacos plásticos, as membranas foram expostas a filmes Kodak X-BRAF QA-S, por 2 a 6 horas. Após procedimento para remoção das sondas, as membranas foram reutilizadas por três vezes até o momento, continuando a apresentar a mesma qualidade de resultados.

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