Chemiluminescent detection of single locus and multilocus hybridization patterns

M. Prinz, C. Loch, M. Staak

Institute for Forensic Medicine, University of Cologne, Melatenguertel 60-62, D-5000 Köln 30, FRG

INTRODUCTION

The chemiluminescent detection of alkaline phosphatase accelerates the time required for DNA-typing, since the signals emitted during the dephosphorylation of 3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD) can be visible after less than one hour.

The rate of light emission is proportional to the enzyme activity, and that means proportional to the amount of membrane bound alkaline phosphatase. Southern blot detection of low amounts of DNA therefore requires a much longer period of film exposure (compare Giles et al. 1990). Working with digoxigenin labeled probes which require blocking and antibody binding steps, these prolonged exposure times of 18 hours and more can cause severe problems with unspecific background.

We compared several modifications of the alkaline phosphatase detection with AMPPD and their effect on unspecific background and sensitivity. Working with multilocus probes, we compared the quality of the band pattern after chemiluminescent detection with the colorimetric detection.

METHODS

Up to four DNA-dilution series were run on the same gels. After Southern blotting and hybridization the membranes (Hybond N, Amersham) were divided and treated according to each modification. The single locus DNA probe used in this study was digoxigenin labeled YNH24 purchased from Promega corporation. As a multilocus probe bacteriophage M13 was labeled using the "Non-radioactive DNA Labeling and Detection Kit" from Boehringer Mannheim. The X-ray film was Agfa Curix RP1 NIF 100. Further details are given in the descriptions of the photos.

RESULTS

The influence of hybridization solutions both with and without formamide was tested for different detection protocols. In each case, after two hours of exposure, the blots hybridized with a 50% formamide solution gave more intensive signals (figure 1, A). After overnight exposure the formamide membranes showed substantial unspecific background (figure 1 C).

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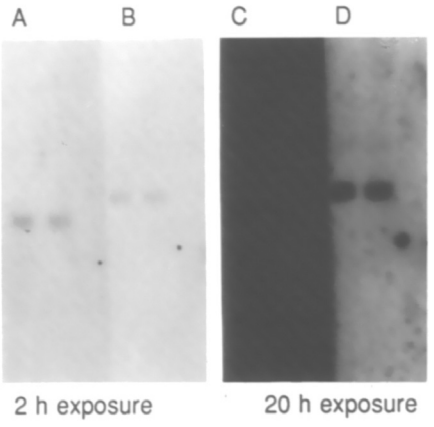

Fig 1. Effect of formamide in the hybridization solution. YNH24 signals of 5µg DNA after 2 and 20 hours exposure to X-ray film. Probe concentration was 2ng/ml. Here detection was performed using maleate buffer, 1% blocking in the antibody dilution and 0.235mM AMPPD in tris-buffer pH9.5
A: hybridization solution 50% formamide and 5% Boehringer blocking reagent
B: no formamide and 0.5% blocking

According to ALLEFS et al. (1990) and the updated manufacturers instructions by Boehringer Mannheim, unspecific background can be reduced by diluting the anti-digoxigenin/alkaline phosphatase conjugate in a buffer containing Boehringer blocking reagent. We tested this to see if it would not impair the sensitivity of the detection assay. DNA-dilution series from 1µg to 10ng DNA were blotted and treated as described in figure 2 A,B. The background is reduced for the antibody/blocking procedure, while the sensitivity is the same. In both cases the 3.0kb fragment could be detected among 25ng DNA. With a DNA content of a human diploid cell of 6.2pg and with the value of 1pg corresponding to 0.965×10^9 bp (Lewin 1987) the heterozygote single copy 3.0kb band in the 25ng track represents a 0.012pg target sequence. For the smaller 1.5kb fragment this amount of target sequence is contained in 50ng (lane 7). The hybridization pattern for the smaller fragment corresponds to this detection limit.

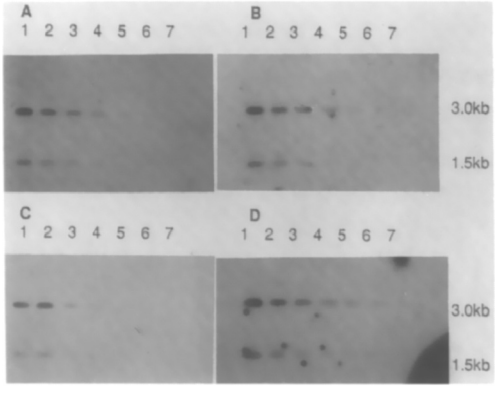

Fig 2. Influence of different detection buffers. Probe concentration of YNH24 was 4ng/ml. A: digoxigenin antibody conjugate was diluted in maleate buffer (0.1M maleic acid, 0.15M NaCl, pH 7.5 adjusted with NaOH)
B: antibody conjugate diluted in maleate buffer containing 1% blocking reagent
A and B: AMPPD diluted to 0.235mM in tris-buffer pH9.5 (0.1M TrisHCl, 0.1M NaCl, 0.05M $MgCl_2$), film exposure 20h
C: antibody-conjugate diluted in maleate buffer 1% blocking, AMPPD diluted in carbonate buffer pH 9.5 (0.05M $NaHCO_4/Na_2CO_4$, 0.001M $MgCl_2$), 20 h exposure
D: as C after 72h exposure

Comparing the alkaline buffers for the dilution of AMPPD, e.g. as the pH 9.5 carbonate buffer used by Tropix Inc. to the pH 9.5 tris-buffer used by Boehringer Mannheim, the carbonate buffer showed a much lower rate of background but also a decreased, or at least slower light emmission rate (figure 2 C). In comparing blots B and C of figure 2 it is noticeable that after the 20h exposure to X-ray film the signal intensity and sensitivity of the assay using tris-buffer is higher. Only after prolonged exposure times can the carbonate assay reach the same sensitivity (figure 2 D).

To test the influence of enhancer molecules DNA-dilution series were incubated for 5 minutes in AMPPD diluted in carbonate buffer, AMPPD diluted in carbonate buffer 10% EMERALD enhancer, and in LUMIPHOS 530 reagent which contains AMPPD, cethyltrimethylammoniumbromide and aminofluorescein (Carlson et al. 1990). The addition of EMERALD enhancer produced dark background, where as there was no difference in background between the AMPPD solution and the Lumiphos 530 reagent. Using Lumiphos 530 the sensitivity was increased.

Prolonging the incubation of the membrane with AMPPD substrate from 5 min to 1h produced higher background and did not improve the sensitivity.

The hybridization of parallel blots with digoxigenin-labeled M13 showed that with the colour substrate developement the bands were sharper and therefore the separation of distinct fragments was improved. The disadvantage of the colour precipitation is the difficult reprobing of the membranes, but for certain applications where no rehybridization is required the colorimetric detection can still be the preferable method.

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