OPTIMIZATION OF THE DIGOXIGENIN/CHEMILUMINESCENCE METHOD FOR THE VNTR DETECTION

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INTRODUCTION

The non-isotopic digoxigenin method provides a feasible alternative to the radioactive-system for the human VNTR determination. Initially, the digoxigenin probes were used in conjunction with colorimetric detection of DNA fragments [1, 2].Then, it was shown that direct chemiluminescent detection enhanced the sensitivity. Furthermore, reprobing was easier with this technique since no colorimetric precipitate needed to be removed [1, 3, 4, 5]. On the other hand, direct chemiluminescence with AMPPD seemed to be more efficient and more flexible compared to luminol-based enhanced chemiluminescent system [6]. However the remaining problem of limited sensitivity is caused by the background from non specific binding of the anti-digoxigenin-alkaline phosphatase conjugate [7].
The aim of our investigation was to improve the resolution of the VNTR band patterns detection. In this respect, we have optimized parameters such as capillary transfer, fixation, hybridization, stringency washes and detection.

METHOD

3 µg saline-extracted genomic DNA was digested with Hinf I (5U/µg) and size-fractionned on a 0.8 % agarose-gel (Seakem-Gold) for 20h at 35V. It was then transfered by capillary blotting to charged nylon membranes (Sigma) and hybridized with 12 ng/mL digoxigenin-labeled pYNH24 probe (Promega). The DNA-fragments were detected with a chemiluminescent system (AMPPD or CSPD - Tropix).

Parameter optimization :

Capillary transfer to charged nylon membranes (Sigma) with Quick-draw blotting paper (Sigma) for 2h in :

- NaOH 0.4 M
- 10xSSC
- NH_4Cl 1M

Fixation :

- UV 245 nm for 15 or 30 sec
- UV 302 nm for 2 min
- baking at 80 °C for 30 min
- UV 302 nm for 2 min and baking at 80 °C for 30 min after a brief alkaline denaturation step followed by neutralization

Hybridization at 68 °C for 20h in 5xSSC / 0.1% N-Lauroylsarcosine / 0.02% SDS with 2.5% I-Light blocking reagent (Tropix) and

- 5% dextran sulfate or,
- 2%, 4%, 6% PEG

Stringency washes in :

- 2xSSC / 0.1% SDS, twice for 5 min at room temperature and 0.1xSSC / 0.1% SDS twice for 15 min at 68 °C
- 2xSSC / 0.1% SDS, twice for 5 min at room temperature and 0.5xSSC / 0.1% SDS twice for 15 min at 68 °C
- 1xSSC / 5% SDS, twice for 20 min at room temperature

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Chemiluminescent detection with 0.1 mg/mL AMPPD in :
- 0.05M $Na_2CO_3/NaHCO_3$ and 1mM $MgCl_2$ at pH 9.5
- 0.1M Diethanolamine (DEA) / 1mM $MgCl_2$ and 0.02% NaN_3 at pH 10 with and without enhancer substances as albumin and PEG

Chemiluminescent detection with 0.1 mg/mL CSPD (Tropix) in 0.1M DEA buffer at pH 10

RESULTS AND DISCUSSION

Capillary transfer (2h) in 10xSSC with Quick draw blotting paper (Sigma) resulted in the maximal signal/noise ratio. Alkali transfer seemed to be incompatible with digoxigenin-YNH24, and NH_4Cl transfer generated a greater background.

Cross-linking at 302 nm enhanced the signal of the bands with respect to baking at 80 °C and decreased the background with respect to UV at 245 nm [8, 9]. It is important to determine the amount of irradiation required in order to prevent the overirradiation of the membrane and to produce the maximum hybridization signal.
Alkali treatment after UV and baking fixation decreased the sensitivity and increased the background (Fig.1).

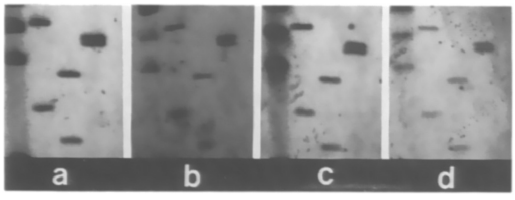

Fig. 1. Cross-linking:

a: at 302 nm
b: at 302 nm after alkali treatment
c: at 80 °C
d: at 80 °C after alkali treatment

Hybridization achieved with the I-light blocking reagent (Tropix) produced optimal results. Hybridization with 5% dextran sulfate reduced background and sensitivity. Hybridization with 2% and 4% PEG decreased the resolution, while using 6% PEG, the sensitivity as well as the background were increased (Fig.2). The greater sensitivity can be attributed to the volumetric exclusion of the probe from the polymer solution and so their effective concentration is increased. On the other hand, a viscous solution can produce a high background [10].

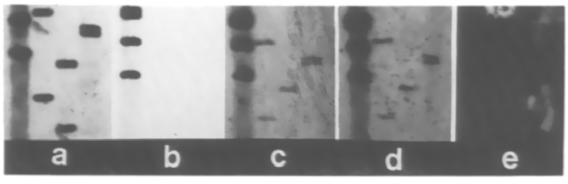

Fig. 2. Hybridization at 68 °C for 20h in 5xSSC and 2.5% I-Light blocking reagent : **a**) with 0.1% N-Lauroyl-sarcosine and 0.02% SDS, **b**) with 2.5% dextran sulfate, **c**) with 2% PEG, **d**) with 4% PEG, **e**) with 6% PEG

Post-hybridization washing with 1xSSC and 5% SDS, twice for 20 min at room temperature gave the best resolution. The background was greatly reduced, and consequently film exposure of 16h and more enhanced the signal of the bands against a relatively clear

background. On the other hand washing with 0.5xSSC at 68 °C resulted in almost the same sensitivity but against increased background. In general, the washing conditions should be as stringent as possible [10]. In our assays, we have determined empirically that with low stringency washes (1xSSC instead 0.1xSSC at room temperature) and a higher SDS concentration (5% instead 0.1%), maximal sensitivity without additional bands, and lower background were obtained (Fig.3).

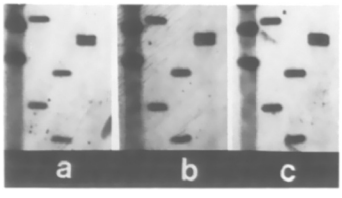

Fig. 3. Stringency washes in :
a) 2xSSC / 0.1%SDS, twice for 5 min at RT and 0.1xSSC / 0.1% SDS twice for 5 min at 68 °C
b) 2xSSC / 0.1% SDS, twice for 5 min at R.T. and 0.5xSSC / 0.1% SDS, twice for 15 min at 68 °C
c) 1xSSC / 5% SDS, twice for 20 min at RT

Membrane blocking : increasing the blocking reagent concentration to 2% (instead 1%), eliminating the washing step between the blocking and antibody binding step and diluting the anti-digoxigenin antibody into the blocking solution improved the sensitivity, decreased the noise and thus allowed a longer exposure time.

Chemiluminescent detection with AMPPD (Tropix) in 0.1M DEA buffer at pH 10 significantly increased the sensitivity compared to carbonate buffer at pH 9.5 [11] and we could detect quantities as small as 60 ng of genomic DNA (Fig.4). Furthermore, the addition of 1% albumine seemed to slightly reduce the background with both carbonate and DEA buffers.

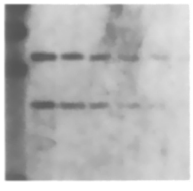

Fig. 4. Genomic DNA titration detected with digoxigenin-labeled pYNH24 and AMPPD (16h film exposure)
From left to right : 2 µg, 1 µg, 500 ng 250 ng, 125 ng, 60 ng

Chemiluminescent detection with CSPD (Tropix) in 0.1M DEA buffer at pH 10 showed a greater resolution of DNA VNTR-bands in comparison to AMPPD at the beginning of the light-emission (1-3 h after substrate addition) while the genomic patterns were stronger with AMPPD after 16-20h of substrate addition and the lambda-DNA bands were more diffuse (Fig.5). These findings corresponded to results already reported [12].

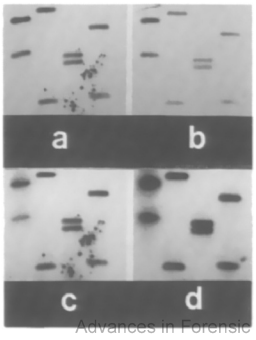

Fig. 5. Chemiluminescent detection
a) with CSPD after 2h.
b) with AMPPD after 2h
c) with CSPD after 16h
d) with AMPPD after 16h

CONCLUSION

The digoxigenin-chemiluminescence is a simple and convenient method for VNTR determination with a sensitivity limit up to 60 ng genomic DNA insofar as the critical parameters previously described are optimized. In this respect, we actually use the following parameters for the detection of the D2S44 locus with digoxigenin-labeled pYNH24:

- capillary transfer to charged nylon membrane Sigma in 10xSSC
- cross-linking at 302 nm for 2 min
- hybridization at 68 °C for 20h. in 5xSSC with 0.1% N-Lauroyl-sarcosine / 0.02%SDS and 2.5% I-light blocking reagent
- two low stringency washes in 1xSSC and 5% SDS for 20 min each at RT
- membrane blocking in 2 % I-light blocking reagent
- dilution of anti-digoxigenin antibody in blocking reagent
- no washing step between the blocking and antibody binding step
- chemiluminescent detection with 0.1 mg/mL AMPPD or CSPD in 0.1M DEA buffer at pH 10

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