EVALUATION OF HYBRIDISATION EQUIPMENT FOR USE WITH NON ISOTOPICALLY LABELLED PROBES

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INTRODUCTION

One of the most critical stages in producing a DNA fingerprint or DNA profile is hybridisation of the membrane-bound DNA samples to a labelled multilocus or single locus (VNTR) probe. Until the early 1990's the probes used were usually prepared by radioactive labelling of purified plasmid inserts. More recently non-isotopic labelling systems have become available and probes for DNA fingerprinting and DNA profiling can now be purchased labelled and ready to use eg, NICE™ probes (Cellmark). Apart from their obvious safety and environmental advantages, non-isotopic probes allow reduced labour costs and more reproducible, high quality results and have therefore been very widely adopted (Ref. 1).

Non-isotopic probes are usually enzyme-labelled oligonucleotides. The high concentration of the oligonucleotide allows very rapid hybridisation when compared with radioactively labelled insert probes, typically 20-30 minutes at 50°C compared with overnight at 65°C. Because the hybridisation reaction is so short, there is insufficient time for temperature equilibration and this can result in incorrect hybridisation temperatures and therefore incorrect stringencies. If probe hybridisation is carried out at too low a stringency, non-specific binding and secondary bands will occur. At too high a temperature the reaction conditions will be too stringent, resulting in loss of sensitivity. It is essential that all solutions are pre-warmed to 50°C, but even then they can cool rapidly as they are transferred into the hybridisation apparatus so that correct hybridisation conditions are never actually achieved, leading to inconsistent quality. To avoid these problems it is important to use a hybridisation system which allows temperature to be maintained exactly throughout the process. A wide variety of hybridisation equipment is now available and several systems were assessed for use with NICE™ probes.

METHODS

The following equipment was evaluated: Hybritube™ (Life Technologies Inc), rotisserie hybridisation oven (Hybridiser 600 Stratagene), NICE™ chamber (Cellmark), sandwich boxes or perspex hybridisation chambers in either waterbaths or dry incubators. Membranes (Hybond-N, Amersham) containing human genomic DNA digested with HinfI were hybridised with NICE™ probes (Cellmark) using recommended conditions. Membranes were sprayed with Lumi-Phos® 530 (Lumigen Inc) and exposed to X-ray film (Hyperfilm, Amersham).

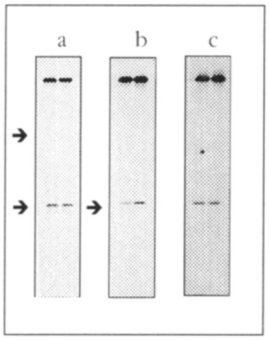

Figure 1
Three identical membranes containing 2 µg K562 DNA digested with HinfI and probed with NICE™ G3. Hybridisation and washing were carried out at a) 40 °C, b) 45 °C and c) 50 °C (recommended temperature). Exposures were for 3 hours at 30 °C.

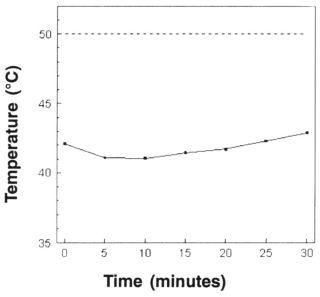

Figure 2
Temperature drift in a hybridisation reaction. Hybridisation solution was pre-warmed to 50 °C then poured into a perspex hybridisation chamber containing membranes and incubated in a 50 °C dry incubator for 30 minutes. The temperature of the solution was recorded with a Squirrel Data Logger (Grant Instruments).

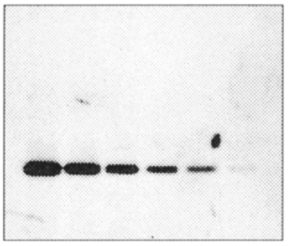

Figure 3a

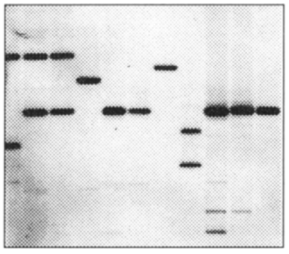

Figure 3b

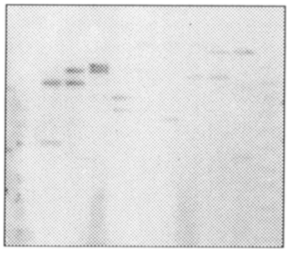

Figure 3c

Figure 3a-c
Membranes hybridised and washed in a rotisserie oven set at 50 °C (a and b) and a Hybritube™ incubated in a waterbath set at 50 °C (c).
a Concentration gradient of HinfI digested K562 DNA probed with NICE™ MS8, 3 hour exposure.
b Human genomic DNA (approximately 1-4 µg per track) digested with HinfI and probed with NICE™ MS8, 3 hour exposure.
c Human genomic DNA (approximately 1-4 µg per track) digested with HinfI and probed with NICE™ MS205 and MW100, overnight exposure.

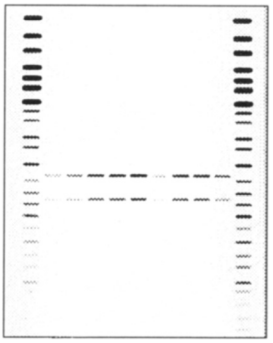

Figure 4
Mebrane containing HinfI digested K562 DNA (2 µg per track) and DNA Analysis Marker Ladder (LTI) hybridised in NICE™ chamber at 50 °C to MS205 and MW100, 3 hour exposure.

RESULTS

Figure 1 shows the effect of deliberately varying hybridisation and washing temperature. Secondary bands are visible when the temperature is below the recommended level. Figure 2 shows how even pre-warmed hybridisation solutions can cool rapidly, leading to hybridisation at temperatures below those intended. Hybridisation temperature fell as low as 41°C reaching a maximum of only 42.9°C after 30 minutes. Figures 3 and 4 show results using various hybridisation equipment. Figures 3a and 3b show membranes hybridised and washed in a rotisserie oven with poor results due to insufficient washing. In Figure 3a the membrane had overlapped and stuck to itself in the roller preventing wash solutions from reaching all areas. Figure 3b shows the middle membrane of three which were hybridised in a single roller. Again efficiency of washing was very poor resulting in high background and secondary bands. The membrane in Figure 3c was hybridised and washed in a Hybritube™ (diameter approximately 20 mm). The results are very patchy with secondary bands and poorly washed areas. The membrane must be rolled quite tightly before insertion into the Hybritube™ and this resulted in poor access for the probe and wash solutions due to the membrane sticking to itself. The membrane in Figure 4 was hybridised and washed in a NICE™ chamber which is specially designed for use with alkaline phosphatase labelled probes. The hybridisation reaction and washing steps occur in a chamber which is surrounded by a waterjacket, allowing very accurate maintenance of temperature throughout the process. Membranes are laid flat in the chamber which is placed on a shaking platform, allowing thorough agitation and ensuring that the probe and wash solutions are in good contact with all areas of the membrane.

DISCUSSION

In order to achieve consistently good results with alkaline phosphatase-labelled oligonucleotides probes it is essential to maintain the correct temperature in the actual hybridisation reaction and subsequent wash steps and to agitate the membranes to prevent sticking and allow thorough washing. Rotisserie ovens are convenient when probing only one or two membranes, but care must be taken to avoid membranes sticking together and to ensure that the solutions do not cool below the recommended temperatures at any stage. Artifacts can be avoided by increasing the amount of mixing, the volume of solution and the diameter of the roller. Significant problems can be encountered when incubating sandwich boxes in dry ovens due to poor heat exchange and cooling of pre-warmed solutions. Results are generally better when incubation is in a shaking waterbath. The most consistent results were obtained using the NICE™ chamber. This system is ideal for probing up to ten membranes simultaneously but probably less appropriate for small-scale hybridisations.

REFERENCES

1. A F Giles, K J Booth, J R Parker, A J Garman, D T Carrick, H Akhavan, A P Schaap. Rapid, simple Non-Isotopic Probing of Southern Blots for DNA Fingerprinting. In Advances in Forensic Haemogenetics 3. Eds H F Polesky and W R Mayr. Springer-Verlag Berlin Heidelberg 1990.