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Non-radioisotopic differential display method to directly visualize and amplify differential bands on nylon membrane

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Differential display is a breakthrough in gene isolation methods (1,2). Compared with other gene isolation methods, it is powerful and relatively rapid. The method achieves isolation of differentially expressed genes by using four 3′-end degenerate primers and 20–40 5′-end arbitrary primers to perform RT–PCR. The PCR products are separated on a DNA sequencing gel and the differences between two or more cell lines are visualized and compared on an X-ray film. To isolate the differential bands from other closely spaced bands on a DNA sequencing gel, one must hold the X-ray film in one hand and cut the bands invisible to the eyes from a transparent gel or filter paper using the X-ray film as a reference. A misjudged band excision wastes days of effort and causes frustration in the subsequent Northern hybridization. To avoid unnecessary frustration and health hazardous radioisotopes, we have developed a non-radioisotopic method to display differentially expressed cDNA bands.

To illustrate the method, a series of human lung adenocarcinoma cell lines (CL1, CLl-2 and CL1-5) with different invasive capabilities are used. Cells are collected by centrifuging at 300 *g* for 10 min and total RNA is extracted by using RNAzol B (Biotecx Laboratories, TX). The mRNA differential display method is performed essentially as described by Liang *et al.* (2) but with some modifications. Five µg of total RNA extracted from cell lines are incubated at 65°C for 15 min before reverse transcription is carried out. 100 pmol of digoxigenin (DIG) conjugated poly-T degenerate primers, e.g., DIG-T_{12}VG, are reverse transcribed in the presence of 0.5 mM dNTP, 10 mM DTT, 0.5 U/µl RNasin (GIBCO-BRL; Gaithersburg, MD), and 200 U of MMLV reverse transcriptase (GIBCO-BRL; Gaithersburg, MD). Reverse transcription is performed at 45°C for 60 min and is stopped by heating to 95°C for 5 min. The sample is then chilled on ice and diluted to 200 µl final volume.

To amplify DNA, 2 µl of the 200 µl sample is subjected to a 40-cycle PCR amplification with the annealing temperature set at 38°C. After PCR amplification, samples are separated on a 6% SDS–polyacrylamide gel and transferred onto a nylon membrane using a semi-dry electro-blotting method. Before electro-blotting, the gel with one glass plate still attached is soaked in a transfer buffer (80 mM Tris–HCl, 120 mM borate, 2.5 mM EDTA, pH 8.3) for 30 min. The gel is then attached to a 3 mm filter paper and made into a transfer sandwich (3). The cDNA bands are electro-blotted onto a nylon membrane at a constant current of 220 mA for 1 h using a semi-dry electro-blotter (Hofer,

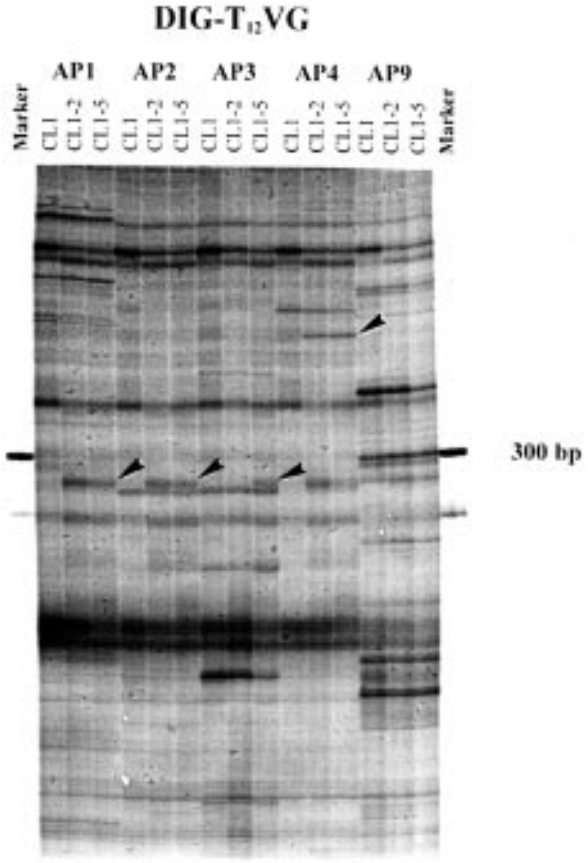

Figure 1. Non-radioisotopic differential display of three tumor cell lines with different invasive capabilities on a piece of nylon membrane. The primer sets used to generate the cDNA bands are shown on the top of the figure. The arrow heads indicate the differences between the three cell lines.

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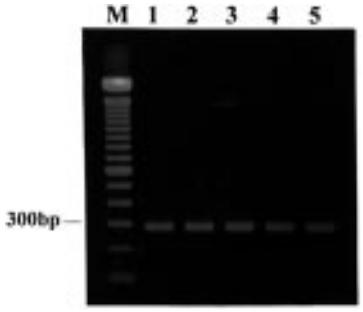

Figure 2. Direct PCR amplification of DNA molecules on a piece of nylon membrane. Lane M contains 100 bp ladders. Lanes 1–5 are serial reamplifications from the same piece of nylon membrane using the primer set DIG-T_{12}VG and AP3.

Semi-Phor TE77). After electroblotting, the membrane is blocked in a 1× BM blocking reagent (Boehringer Mannheim; Mannheim, Germany) for 30 min at room temperature.

To detect DIG labeled cDNA bands, the membrane is incubated with anti DIG-alkaline phosphatase antibody/enzyme conjugate (Boehringer Mannheim; Mannheim, Germany), diluted 10 000-fold in a dilution buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20, pH 7.5) containing 0.5× BM blocking reagent, for 45 min at room temperature. The membrane is then washed three times with the dilution buffer for 10 min each time. A signal is generated by alkaline phosphatase colorimetric reaction using 0.42 mg/ml NBT and 0.21 mg/ml BCIP in substrate buffer (0.1 M Tris–HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$). The reaction is carried out in a polypropylene bag or box for 30 min and terminated by rinsing the membrane in PBS buffer solution containing 1 mM EDTA. Several differentially expressed bands can be observed on the membrane and are indicated by the arrow heads in Figure 1.

The bands of interest can be excised from the membrane. The samples on the excised membrane strips can be directly reamplified by PCR after rinsing the membrane briefly with 1× PCR buffer. To illustrate, one of the differential bands generated by the primer set of DIG-T_{12}VC and AP3, has been cut out from the membrane and reamplified directly without any purification. The small piece of membrane containing the DNA sample can be preserved at room temperature for months, if not years, and can serve as a PCR template at least five times, as shown in Figure 2. Since DNA amplification can be achieved directly from the membrane strip, it is convenient for differential display procedures such as cloning, sequencing and probe preparation.

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