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Differential screening of gene expression difference enriched by differential display

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Since its original description in 1992 (1), differential display has become a widely used methodology for cloning differentially expressed genes. Much effort has since been made to further optimize and streamline the technique (2,3). However, one major bottle neck remaining for the method lies at the screening step for the verification of the cDNA fragments identified by differential display.

The standard protocol recommends the use of reamplified cDNA directly as a probe for Northern blot verification (4). The other approach is to clone the reamplified cDNA and then use it as a probe. Both approaches have their pros and cons. It is known that the reamplified cDNA probes from differential display sometimes contain a mixture rather than a single cDNA species, due to the contamination of other co-migrating cDNA fragments. As a result, direct use of the cDNA reamplified may be problematic because multiple messenger RNA species may be detected, making the identification of the truly differentially expressed gene difficult. On the other hand, if the cDNA probe is cloned, the choice of which clone to use as a probe becomes a matter of luck depending on the extent of cDNA contamination.

Compounding the problem is the laborious screening by Northern blot for each cDNA fragment identified or purified. It is especially difficult under circumstances where the amount of RNA samples is limited. To this end, various approaches have been tried to improve the method (5).

Here we describe the use of differential display followed by differential screening, using 'Reverse Northern' and colony hybridization to screen for cDNA fragments that truly represent differentially expressed mRNAs. The method takes advantage of the high cloning efficiency of the pCR-TRAP cloning system, which is based on positive-selection for inserts. Reamplified cDNA products from differential display are ligated with the pCR-TRAP vector and only recombinant plasmids confer antibiotic resistance. Antibiotic resistant colonies are transferred onto duplicate filters, which are then probed with cDNAs labeled by reverse transcription from the 'plus' and 'minus' RNA samples.

As part of our ongoing effort to apply differential display to identify genes that are regulated by the Ras signaling pathway, the immortalized rat embryo fibroblast cell line Rat1 and its H-ras transformed derivative, T101-4 (6) were compared. Differential display was performed using the RNAimage kit (GenHunter, Nashville, TN) essentially as instructed. The cDNA fragments of interest (Fig. 1A) were retrieved from the denaturing polyacrylamide gels and reamplified with the same sets of primers as in the initial differential display PCR reactions. Five microliters of the PCR products were directly ligated with 2 µl pCR-TRAP vector (GenHunter, TN) as instructed. The ligated plasmids were transformed into GH competent cells (GenHunter, TN) and plated on LB plates containing 20 µg/ml tetracycline. After an overnight incubation at 37°C, 50–200 tetracycline resistant colonies were obtained with each cDNA insert, while the vector self-ligation gave no colonies (data not shown).

Reverse Northern dot-blot was performed initially to test the feasibility of differential screening. Specifically, four tetracycline-resistant colonies were randomly picked from each plate and lysed by boiling in 50 µl lysis buffer consisting of 0.1% Tween 20 in TE buffer, pH 8.0. Using the primers that flank the cloning site of the vector, the cloned cDNA fragments were amplified from the colony lysates (Fig. 1B). As can be seen, most of the colonies analyzed contained inserts of the expected sizes.

To prepare for blotting, 30 µl of each PCR-amplified cDNA insert from Figure 1B was mixed with 10 µl 2 N sodium hydroxide, denatured by boiling for 5 min, and then neutralized with 10 µl 3 M sodium acetate, pH 5.0. After bringing the total volume to 110 µl with dH_2O, 50 µl of each sample was dot blotted onto duplicate Nylon membranes using the Bio-Dot microfiltration system (BioRad, CA). The membranes were UV cross-linked and rinsed in 6× SSC before being used for hybridization (4).

The cDNA probes were prepared from 10–50 µg of each of the two RNA samples by reverse transcription in a 50 µl reaction which consists of 25 mM Tris–HCl, pH 8.3, 38 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT, 30 µM dNTP (without dCTP), 1 µM T_{20} primer and 25–100 µCi [α-^{32}P]dCTP (3000 Ci/mmol; NEN, MA). After a 5 min incubation at 65°C, the samples were shifted to 37°C and 1000 U MMLV reverse transcriptase (GenHunter, TN) was added, followed by continued incubation for 1 h. After the reverse transcription, Quick Spin column (Boehringer Mannheim, IN) was used to remove the unincorperated ^{32}P. Equal counts ($5–10 \times 10^6$ c.p.m.) of the cDNA probes from Rat1 and T101-4 were heat denatured and used to probe the duplicate blots.

The results from the dot-blot experiment are shown in Figure 1C. Mob-5, a previously cloned gene expressed only in the transformed cells (Liang *et al*., unpublished result), was used as a positive control. Both clones of mob-5 showed signals with the cDNA probe from T101-4 cells but not from the Rat1 cells, as expected. As a negative control, two clones from a band equally expressed from the differential display (Fig. 1A) gave equal signals on dot blots for both cDNA probes, indicating equal probe labeling. Three of the Mob-36 clones showed quantitative difference between the two cell lines, with the forth clone (Mob-36d) being a false positive. Mob-37, Mob-38 and Mob-39 each had 3, 2 and 4 clones scored positive, respectively. Mob-37d (no insert) and

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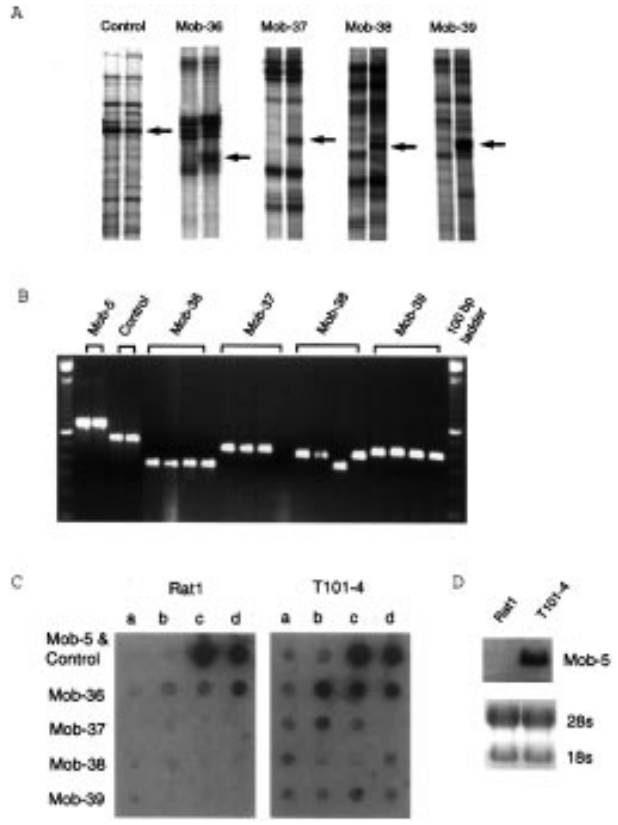

Figure 1. (**A**) Differential display of the messenger RNA from Rat1 cells (left lanes) and T101-4 cells (right lanes). cDNA fragments of interest were indicated by arrows. H-T_{11}C was used as the anchor primer in combinations with arbitrary primers H-AP18, H-AP18, H-AP29, H-AP31 and H-AP34 (GenHunter, TN) to amplify the negative control, Mob-36, Mob-37, Mob-38 and Mob-39, respectively. (**B**) 1.5% agarose gel electrophoresis of the cloned cDNA. Two randomly picked tetracycline resistant colonies for the positive control, Mob-5 and the negative control, four randomly picked tetracycline colonies for each of the Mob-36, Mob-37, Mob-38 and Mob-39 were amplified by PCR with a pair of primers flanking the cloning site of the pCR-TRAP cloning vector (GenHunter, TN). (**C**) The colony PCR products from (B) were blotted on duplicate filters as indicated, and probed with ^{32}P-labeled cDNAs from either Rat1 (left blot) or T101-4 (right blot) cells. For the first row, dots a and b represent two clones from the positive control Mob-5, while dots c and d represent the negative control. (**D**) Northern blot analysis of Mob-5, showing the differential expression of the gene in H-ras transformed T101-4 cells. The 28S and 18S rRNAs are shown at the bottom as loading control.

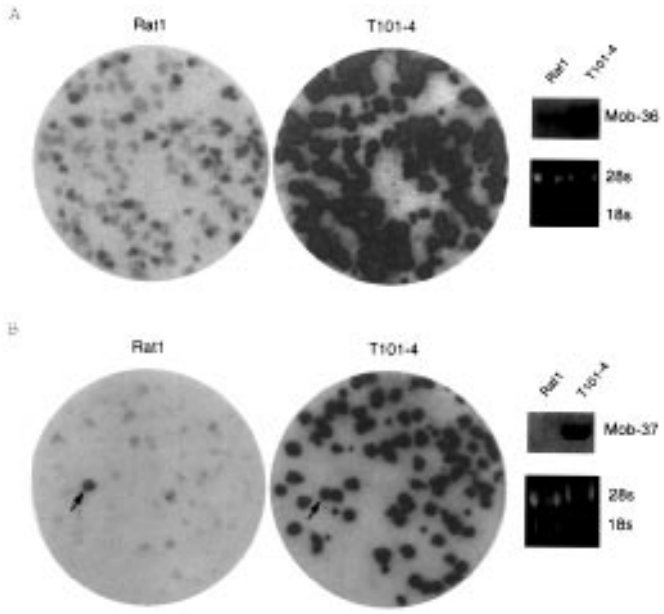

Figure 2. Differential screening of cDNA fragments obtained from differential display. After reamplification, Mob-36 and Mob-37 were each cloned into the pCR-TRAP cloning vector. Tetracycline-resistant colonies from Mob-36 (**A**) and Mob-37 (**B**) were transferred onto duplicate filters and processed for differential hybridization with labeled cDNA probes from Rat1 cells or T101-4 cells. The arrow denotes a false positive clone from Mob-37. Northern blot analysis (right panels), use the cloned cDNA probe from differential screening to confirm the differential expression of the genes.

Mob-38b and Mob-38c were negatives, which could represent contaminating genomic DNA.

To further streamline the screening procedure, differential screening by colony hybridization was carried out directly after the pCR-TRAP cloning. Tetracycline-resistant colonies were transferred by replica-plating onto duplicate nitrocellulose membrane filters. After an overnight incubation, the filters were floated in 0.5 N NaOH/1.5 M NaCl for 2 min to lyse the cells and denature the DNA, then neutralized with 0.5 M Tris–HCl buffer (pH 7.0)/1.5 M NaCl. Filters were then washed in 6× SSC plus 0.5% SDS at room temperature for 1 h, with vigorous shaking to remove the cell debris from the filters. After cross-linking by UV irradiation for 2 min, the filters were hybridized with the same set of ^{32}P-labeled cDNA probes used for dot blots. Shown in Figure 2 are representative results of this screening procedure. For both Mob-36 and Mob-37, most of the clones showed differential expression in the transformed T101-4 cells, consistent with the results from dot blot analysis. Using positive clones thus identified as probes, Northern analyses confirmed the validity of this screening technique (Fig. 2, right panels). It is worth noting that Mob-36, which appeared to be a quantitative difference between the filters, also exhibited quantitative difference on Northern blot (Fig. 2A). One false positive colony was detected for Mob-37 (indicated by the arrow, Fig. 2B).

Here, differential screening has been used to easily and rapidly identify the differentially expressed messages enriched by differential display. The method has the following advantages: (i) many filters representing different cDNA fragments from differential display can be screened with the same set of probes; (ii) the masking effect of false positive clones can be resolved; (iii) once a cDNA clone is confirmed to be positive, it is ready to be sequenced, checked against the database and, if necessary, used as a probe to screen libraries.

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