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Chemical cross linking subtraction (CCLS): a new method for the generation of subtractive hybridisation probes

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Submitted March 25, 1992

The cloning of differentially expressed genes by subtractive hybridisation,is a widely used technique that usually involves an eco-physical separation (hydroxyapatite, avidin-biotin) of the common cDNA-RNA hybrids from the unique single stranded cDNA (1, 2, 3).The published methods often lead to incomplete separation of these components and to a significant loss of probe material, including the low abundance copies of potentially interesting genetic information (4). In all these methods the radiolabelled probe requires manipulation throughout the procedure. We have developed a novel, one tube, protocol based on specific chemical cross linking of the cDNA-RNA hybrids which requires no physical separation and overcomes most of these problems.

The example we describe uses RNA isolated from mitozolamide treated RJKO cells. This treatment induces expression of the O^6-methylguanine methyltransferase gene (O^6-MMT) (5) but this is at low abundance when compared with the expression of α-actin RNA transcripts (Figure 1). First strand cDNA was synthesised from 3–5 μg. of poly A^+ mRNA, from induced cells, using 'Superscript' Gibco BRL according to the manufacturers recommended protocol. It was necessary to use this enzyme, in preference to other Avian or murine enzymes, in order to avoid excessive second strand synthesis. RNA was removed from the cDNA by alkaline hydrolysis (0.5 M NaOH, 55°C, 15 min) and passed through a Sephadex G50 spin column. Approximately 500 ng. of this first strand cDNA was hybridised to 10 μg of DNA free poly A^+ mRNA from RJKO cells that do not express the O^6-MMT gene (i.e. not induced by mitozolamide) (5). Hybridisation was carried out for approximately 20 hours at 68°C in a total volume of 10 μl containing 0.5 M NaCl, 25 mM Hepes buffer pH 7.5, 5 mM EDTA, 1% SDS. This mixture was then diluted five fold with sterile distilled water, and the RNA-cDNA complex precipitated by the addition of three volumes of ethanol. The precipitate was dissolved in 50 μl of 25 mM Tris–HCl pH 7, 1 mM EDTA, 5% DMSO, 2 mM Ascorbic acid and incubated at 68°C for 3 minutes to remove hairpin structures from the single stranded cDNA. The incubation temperature was then lowered to 45°C and 2,5 diaziridinyl-1,4-benzoquinone added to a concentration of 200 μM.This was left for 20 minutes during which time all GC pairs of RNA-cDNA hybrids are effectively cross linked (6). This material was subsequently ethanol precipitated and, if required, after addition of a further 10 μg of DNA free poly A^+ mRNA from RJKO cells can be rehybridised for an extra 20 hours as previously described. The subtracted probe was produced by directly labelling the product with 100 μCi of [α^{32}P] dCTP, 3000 Ci/mMol, using random priming (7, 8) except that 6 units of Sequenase II (US Biochemicals) was substituted for Klenow and the incubation carried out for 20 minutes at room temperature. The substitution of Klenow with Sequenase was essential for the selective radiolabelling described. This is due to Sequenase lacking exonuclease activity and being unable to either produce strand displacement or, most importantly, to utilise RNA as a template for radiolabelling (9).

Figure 1 shows a Southern blot (100 ng each) of α-actin and O^6-MMT inserts probed with subtracted (1 cycle) (A) and unsubtracted (B) probes made with equal amounts of cDNA. The results of the unsubtracted probe (B) show that the α-actin gene has an observed signal approximately 120–150 fold higher than the O^6-MMT level of expression. The subtracted probe (A) shows that the α-actin signal has been reduced to half that of the O^6-MMT which was maintained and gives an overall enrichment of 240–300 fold after only one round of hybridisation subtraction (autoradiograph densitometry). The major advantages of this cross linking technique are: 1) the subtracted probe does not need to be radiolabelled until use, thereby reducing manipulation of labelled DNA and also probe decay: 2) more than one probe can be made from one batch of subtracted material; 3) the technique does not invoke physical separation of cDNA-RNA hybrids from unique cDNA's thereby improving efficiency and reducing losses of material.

The technique requires at least 10 μg of poly A^+ RNA which may be a limiting factor for some applications however, this can be overcome by the use of a directional cloning vector such as that described by by Pallazzolo and Meyerowitz (10) whereby sense or antisense RNA can be synthesised from a cDNA population.

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