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Polymerase chain reaction products containing 5-methyldeoxycytidine: a microplate immunoquantitation method

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Numerous non-isotopic techniques have been developed in the last decade to detect and quantitate hybridization products (1). Since the polymerase chain reaction (PCR) was introduced this technique has become increasingly important for the diagnosis of bacterial and viral infections. The need for simple methods allowing quantitative evaluation of PCR products has resulted in the development of alternatives to gel electrophoresis and blotting. The most sensitive techniques rely on the incorporation of biotinylated dUTP or of a digoxigenin-dUTP complex. The products containing the modified nucleotide are recognized by an avidin-enzyme conjugate or an anti-digoxygenin antibody. The use of 5-methylcytidine (5-MeCyd) offers an alternative to the labeling of nucleic acid probes with biotin-dUTP. It is possible to raise antibodies against this hapten. We obtained such antibodies, either polyclonal or monocional, and used them to detect and quantitate the presence of 5-MeCyd in urine samples from cancer patients (2), in metaphase chromosomes (3) as well as in the nuclei of cells grown *in vitro* (unpublished results). Following on from this we tested the possibility of using these antibodies to detect and quantitate products generated through PCR made in the presence of 5-MedCTP. The reaction was carried out in the presence of biotinylated primers, specific for the Epstein–Barr virus (EBV), which allowed the PCR products to be retained in a polystyrene microtitration plate to which avidin had been covalently linked.

Two primers spanning a segment of 186 base pairs were chosen in the BamHIW region of the EBV genome. This is the repeating unit of the long internal direct repeat, IR1 and is highly conserved between the different EBV strains (4). Each primer was biotinylated at the 5'-end during synthesis. They had the following sequences: PER1: 5'TTT GTC CCC ACG CGC GCA TA 3' and PER2: 5'AGG TGG CGT AGC AAC GCG AA 3'. Template DNA for PCR amplification was extracted from the Burkitt's lymphoma cell line Namalwa which does not contain episomes or viruses but where two EBV genomes are closely integrated at a known site of the chromosome 1 (5). Thus, a single Namalwa cell harbors two EBV copies. Serial dilutions of Namalwa cells were carried out in sterile deionised water and used as sources of templates.

The PCR amplification was carried out as follow: aliquots (10 μl) of cell lysates were subjected to DNA amplification in 100 μl reaction buffer, consisting of 50mM KCl, 10 mM HCl (pH 8.4), 1.5 mM MgCl2, 100μg/ml gelatin, 200μM each of dATP, dTTP and dGTP (Perkin-Elmer Cetus), 40 μM 5-Methyl dCTP (Boehringer-Mannheim, Mannheim, Germany) and 40μM dCTP (Perkin-Elmer Cetus), 0.5 μM of each primer, 1 unit of Taq polymerase. Thermal cycling was carried out using a Perkin-Elmer 480 machine as follows: one cycle consisting of 10 min

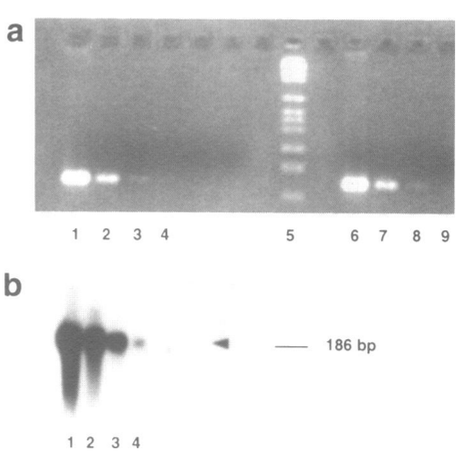

Figure 1.a. Gel electrophoresis of PCR products. Serial tenfold dilutions of EBV DNA ranging from 20,000 to 20 genomic copies per reaction were used as templates. 10 μl of each PCR product was analysed by agarose gel electrophoresis. The products were visualised by ethidium bromide staining under UV. Electrophoresis analysis revealed that the amplified DNAs were of the expected sizes as judged from migration distances (N-PCR: lanes 1–4; Biot/5Me PCR: lanes 6–9). The sensitivity of the reaction as determined by the intensity of the signal on the gel attained 200 target DNA molecules in each PCR system. Lane 5: Molecular weight markers VI 154–2156 bp from Boehringer. **b.** Southern analysis. Amplified products in the left part of the gel shown in Figure 1a were submitted to alkaline Southern transfer and hybridized with a DIG-11 dUTP-labeled EBV probe providing a sensitivity of 20 DNA molecules (lane 1: 20,000 copies; lane 2: 2,000 copies; lane 3: 200 copies; lane 4: 20 copies).

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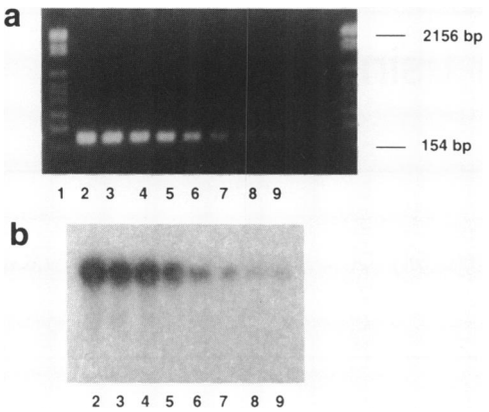

Figure 2.a. Electrophoresis of products obtained with the Biot/5Me-PCR. Serial twofold dilutions of amplified products obtained with Biot/5Me PCR were loaded into each slot of the gel and visualized as for Figure 1a. (Lanes 1 and 10: markers. Lane 2: 1280 fmol). **b.** Immunodetection of 5-MeCyt on blots. After migration the gel shown in Figure 2a was submitted to alkali-denatured Southern transfer. Blots were soaked for 30 min at 20°C in 0.5% blocking reagent (Boehringer) diluted in 0.05M Tris buffered saline pH.7,4 (TBS). They were washed three times in PBST (PBS containing 0.2% Tween 20) and incubated for 1 hour at 20°C with anti-5MeCyd monoclonal antibody (IgG1; non-diluted hybridoma supernatant). After three washings in PBST, blots were incubated for 1 hour at 20°C with peroxidase-conjugated goat anti-mouse IgG (H+L) (BioRad, Richmond CA), diluted 1/100 in PBST, washed three times in PBST and rinsed with TBS. The substrate was then added (60 ml of 0.3% 4-chloro-1-naphtol in methanol in 100 ml of PBS with 0.02% hydrogen peroxide) and incubated at 20°C for 30 min. The limit of detection reached with this method was 10 fmol, which corresponds to 2 copies of the viral genome.

at 99°C, 2 min at 62°C and 2 min at 72°C, followed by 35 cycles of 30 seconds at 95°C, 1 min at 62°C and 2 min at 72°C. Samples were kept at 72°C for 5 min at the end of thermal cycling.

To analyse products from the PCR performed with biotin-labeled primers and 5-MedCTP (Biot/5Me PCR), we compared results obtained after Biot/5Me PCR with those observed in an experiment where unlabeled primers and conventional dNTPs (N-PCR) were used.

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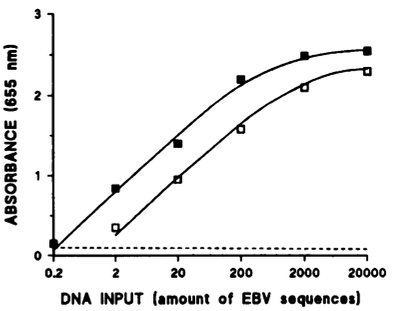

Figure 3. ELISA of PCR products. Avidin coating: 100 µl of 10 mg/ml N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride, (Merck) diluted in pH 7.2 phosphate buffered saline (PBS) was distributed in each well of 96-well carboxylated polystyrene microtier plates (6) and kept at 20°C for 10 min. 100 µl aliquots of avidin (Sigma, St Louis, MI) diluted in PBS (1O µg/ml) were then distributed in the wells. After 1 hour plates were washed once with PBST then three times with PBS. Heat-denaturated PCR products were distributed (50 µl/well) and left for 15 min at 20°C. Wells were then washed three times with PBST. 50 µl of monoclonal antibody anti-5MeCyd (undiluted hybridoma supernatant) was added, incubated for 30 min at 20°C and then washed three times with PBST containing 1% bovine serum albumin (PBST-BSA). 50 µl of peroxidase-conjugated goat anti-mouse IgG (H+L) (BioRad) diluted 1/1000 in PBST was added to each well, left 30 min at 20°C, then rinsed three times with PBST-BSA, prior the addition of the substrate. The peroxidase substrate used (TMB peroxidase EIA substrate kit, BioRad) was 3, 3′ 5, 5′ tetramethylbenzidine (solution A in dimethylformamide), mixed with hydrogen peroxide (solution B) at a ratio of 10/1. 100 µl of this mixture was added into each well and left at 20°C for 10–20 min, until the deep blue reaction product appeared. The absorbance was measured on a microplate reader at 655 nm (BioRad model 2550). When serial dilutions of the methylated PCR products were analysed, the signal intensity increased with the amount of amplified products. The detection limit reached using this procedure was lower than 2 viral genome copies (Fig.3, closed symbols). When PCR was performed with Dig-11-dUTP (40 µm), anti-digoxygenin antibodies (Boehringer Mannheim) were diluted 1/500. The detection limit was 2 copies of viral DNA (Fig.3, open symbols). In the absence of methylated products or with unrelated antibodies a background signal of 0.1 A260 unit was obtained (dashed line).