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

Alain Niveleau¹,²,* Emmanuel Drouet², Cecile Reynaud³, Fouad Fares², Corinne Bruno² and Christiane Pain²
¹CNRS URA 1459, ²Dept. of Virology, Institut Pasteur de Lyon, Avenue Tony Garnier, 69365 Lyon, France and ³School of Medicine, Dept. of Biochemistry, University of Sherbrooke, Quebec, Canada J1H 5N4

Received September 8, 1994; Revised and Accepted November 3, 1994

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 monoclonal, 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urine samples from cancer patients (2), in metaphase chromosomes (3) as well as in the nuclei of cells grown in vitro (unpublished results).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-MeCyd. 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 1a. 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; Bio/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).

* To whom correspondence should be addressed
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-MeCTP (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.

ACKNOWLEDGEMENTS

This work was supported by Ligue Nationale contre le Cancer and Fondation de France.

REFERENCES