

A quantitative HpaII-PCR assay to measure methylation of DNA from a small number of cells

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Methylation-sensitive restriction enzymes and Southern blot analysis are commonly used to assay for DNA methylation, usually requiring DNA from about 10^5 cells. However sensitivity adequate for only a few hundred cells is needed in many cases, for example, the study of DNA methylation changes in germ cells or pre-implantation embryos. We earlier reported a sensitive PCR assay using primers that bracket a HpaII site; if the DNA is treated with HpaII prior to PCR, an amplified product is seen only from a methylated genomic template (1). We report here two improvements which make the assay quantitatively applicable to 100–300 cells, with as little as 10% DNA methylation detectable.

We now include in each reaction an internal standard sharing primer binding sites with the genomic template, but distinguishable by size (2). The inset in Figure 1 shows the PCR products from the internal standard (band # 1), and from the genomic template (female blood DNA) with or without prior treatment with HpaII (band # 2). Also shown in Figure 1 are the densitometric tracings of these ethidium bromide-stained bands. Plotted in Figure 2 is the ratio of the internal standard band (I) to the genomic band (G) in an experiment in which the internal standard was varied from 80 to 240 molecules, while the genomic DNA (500 pg, approximately 100 molecules) was held constant. We find that female mouse spleen and blood DNA are methylated $52.5 \pm 2.3\%$ and $50.6 \pm 3.5\%$, respectively, in agreement with previous data (1). To prepare DNA suitable for the HpaII-PCR assay, we used a guanidine HCl procedure (3), modified as described in the legend to Figure 1.

ACKNOWLEDGEMENT

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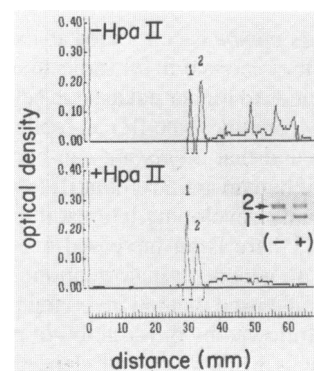


Figure 1. Assay of female mouse blood DNA by HpaII-PCR. Anticoagulated blood ($2 \mu\text{l}$) was mixed with lysis buffer ($161.5 \mu\text{l}$) (3) and $1 \mu\text{g}$ of M13 RF DNA. After heating at 60°C for 1 hr, the DNA was sheared by passage 10 times through a 26 gauge needle. Twenty μg of glycogen was then added, followed by ethanol precipitation at room temperature. After resuspension, the DNA was digested in restriction buffer (4) with XbaI (to reduce DNA size); one half of the reaction mix was then incubated with HpaII at 37°C for 3 hr. The HpaII was completely inactivated by heating the samples to 100°C for 15 min in a heat block. The samples were then diluted into a PCR reaction mix as described (1), but with the addition of 300 molecules of internal standard to each tube. Primers 1 (d[CACGCTTCAAAAAGCGCACGTCT]) and 3 (d[CTTGAGGGCAGCAGT-ACGGAAT]), flanking HpaII site 7 in the 5' region of the X-linked PGK gene, have been described (1). Two preliminary cycles of PCR were done (95°C , 4 min; 60°C , 1.5 min; 73°C for 1 min) to assure complete denaturation of the genomic DNA, followed by an additional 35 cycles (94°C , 1 min; 60°C , 1.5 min; 73°C , 1 min) (thermal cycler: Ericomp). The PCR products were analyzed on a 2.6% agarose gel containing ethidium bromide, and quantified by densitometry (1). Inset shows gel pattern of PCR products without (–) or with (+) HpaII. Upper band, 170 bp PCR product of genomic template; lower band, 133 bp product of internal standard.

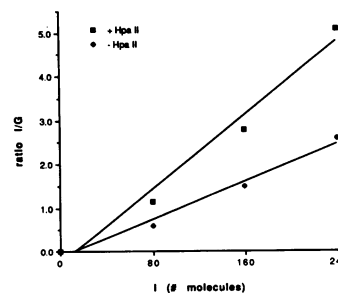


Figure 2. The Hpa II-PCR assay of female mouse spleen DNA. DNA (500 pg), uncut (closed symbols), or digested with HpaII (open symbols), was used for PCR in the presence of the amount of internal standard indicated. The internal standard was synthesized from genomic mouse DNA by PCR with primer 3 and a second primer designed to give a product containing a deletion relative to the genomic template. The 'deletion' primer, d[CACGCTTCAAAAAGCGCACGT-CTCGACCTCACGGTGTGCCC], was identical to primer 1 for the first 22 nt, but had an additional 18 nt corresponding to a sequence located 37 bases downstream of primer 1. G, genomic template; I, internal standard.