Nucleic Acids Research, 1992, Vol. 20, No. 22 **6117–6118**

A simple method for elimination of unspecific amplifications in polymerase chain reaction

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Received July 20, 1992; Revised and Accepted October 20, 1992

The polymerase chain reaction (PCR) is used widely to detect small quantities of selected DNA sequences, including latent infections by viruses and bacteria. A few or even a single copy of viral or 'target' DNA sequences can be amplified *in vitro* to a level detectable by gel electrophoresis or Southern blot hybridization (1). Recently, most efforts have been directed towards improving PCR assays that can act as simple but highly sensitive diagnostic tests for detecting crucial pathogenic/prepathogenic nucleic acid markers of malignancy or other diseases. However, an important difficulty often encountered is non-specific amplification and/or very weak amplification of target DNA irrespective of type of specific oligonucleotide primers used. This has led to enormous interlaboratory variations in PCR results.

Over the past few years, PCR has also been used extensively in cervical specimens to detect human papillomaviruses (HPVs) which have been strongly implicated in the process of cervical carcinogenesis (2, 3). Significant variations have been observed in the PCR positivity of HPV (Most notably types 16 and 18) in precancerous lesions and in the normal population (4), which generally carry a very low level of HPV infection. We report here a simple and rapid method for elimination of unspecific amplication while detecting target DNA fragment by PCR.

The method involves digestion of genomic DNA with an appropriate restriction enzyme which cuts the circular (viral) or high molecular weight genomic DNA outside the region to be amplified and use of this digested DNA for PCR. The major effect of cleavage is perhaps to cut between sites at which primers might bind spuriously. It facilitates not only the elimination of non-specific amplification but also good amplification of the expected region, which can easily be detected by gel electrophoresis. Figure 1 shows PCR amplification of 3 DNA samples from cervical scrapes with and without digestion by a single-cut enzyme for HPV 16, BamHI. There is a complete absence of unspecific bands in the PCR product of digested DNA samples in lanes 4, 6 and 8 as against their undigested counterparts which show unspecific bands in lanes 3, 5 and 7 respectively. Although the reason(s) for an unspecific amplification in undigested DNA is not known, it may be due to primer extension after cross-over caused by supercoiling of circular DNA. Poor or no amplification (see lane 5) may occur if the target DNA is not easily available to the primers. After restriction digestion, circular DNA is linearised and the genomic DNA is cleaved in a way that facilitates easy availability of target DNA to the oligonucleotide primers for annealing. This technique is effective in cases where genomic PCR gives artefacts and increased concentration of Mg++ in reaction buffer (1.5 to 4 mM), time of extension (2 to 5 min) and/or changes in annealing temperature (from 50 to 65°C) do not improve the amplification of a fragment of the correct size. Any enzyme that cuts frequently but does not cut the desired amplification product (AluI) is effective in improving specificity and yield of normal genomic PCR (see Figure 2). In case of circular DNA such as HPV DNA, digestion with a single cut enzyme (BamHI for HPV 16; EcoRI for HPV 18) acts with equal efficiency as with multicut enzymes.

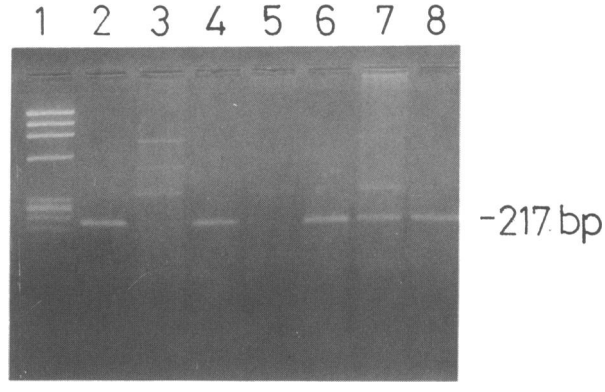

Figure 1. PCR amplification of HPV 16 DNA sequences in undigested and digested DNA of cervical scrapes. 2 μg of each DNA sample was digested with 10 units of BamHI (Boehringer) for 2 hrs. 0.5 μg of digested and undigested DNA were used for PCR. Amplification was carried out using HPV 16-specific oligo primers (p1.5'-AAGGCCAACTAAATGTCAC-3' and P2.5'-CTGC-TTTTATACTAACCGG-3') from its upstream regulatory region (URR) between nucleotides 7763 and 75-(amplimer 217 bp). The method involves 50 μl of reaction mix in 0.5ml Eppendorf tube containing 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, and 75 μg/ml BSA with deoxyribonucleotides at final concentration of 200 μM each and primers at 1 μM and 2.5 U Taq DNA polymerase (Cetus, USA). The reaction mixture was overlayered with 50 μl of mineral oil (Cetus) and the amplifications were carried out in a DNA Thermal Cycler (Perkin Elmer). Cellular DNA was denatured at 94°C for 5 min, annealed at 55°C for 1 min followed by extension for 2 min at 72°C. These steps were repeated for 35 additional cycles with denaturation (94°C) for 1 min only. 15 to 20 μl of amplified product was run in 3% Nusieve–agarose gel, stained with ethidium bromide and photographed under a UV transilluminator. Lanes 1 and 2 are HaeIII digested ΦX174 DNA marker and vector-free HPV 16 DNA respectively.

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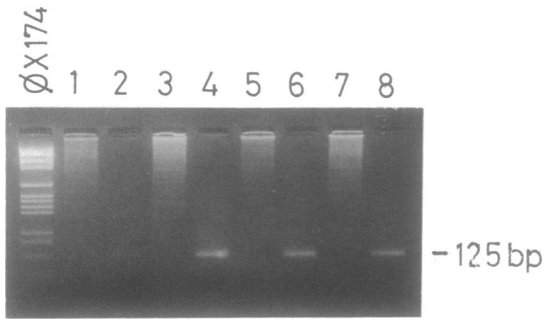

Figure 2. PCR amplification of cellular p53 gene sequences with or without digestion by AluI. Lanes 1, 3, 5 and 7 are undigested cervical carcinoma biopsy DNA showing unspecific amplification, while the corresponding digested DNA in lanes 2, 4, 6 and 8 show specific amplification for the correct size of 125 base-pair fragments. Left-hand lane, marker fragments, generated by HaeIII cleavage of phage ΦX174 DNA.

It is, therefore, suggested that where there is a low copy number of target DNA sequence or circular genome to be detected, the sample DNA should be digested with an appropriate restriction enzyme before processing it for PCR. We have been routinely using this technique in our laboratory for detecting various HPV types (HPV 16, 18, 11, 6, 31, 35) and other gene sequences such as p53, Rb1 c-myc etc. in genomic DNA extracted from scraped cervical cells, blood, urine and biopsies. More than 200 samples have been analysed by this method and the results are found consistent. Restriction digestion thus improves specificity of amplification and increases the efficiency and reliability of PCR in detecting low copy number of target DNA sequences of interest including those of viral/bacterial genomes in clinical samples.

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