A novel method for producing partial restriction digestion of DNA fragments by PCR with 5-methyl-CTP

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ABSTRACT

Partial digestion of DNA fragments is a standard procedure for subcloning analysis and for generating restriction maps. We have developed a novel method to generate a partial digestion for any DNA fragment that can be amplified by PCR. The method involves the incorporation of 5-methyl-dCTP into the PCR product to protect most of the restriction sites. As a result, complete digestion of the modified PCR products with a 5-methyl-dCTP-sensitive enzyme will produce an array of restriction fragments equivalent to a partial restriction enzyme digestion reaction done on unmethylated PCR products. This method reduces the time and material needed to produce partially-digested DNA fragments by traditional methods. Furthermore, using fluorescein-labeled primers in the reaction, we were able to detect the fluorescein-labeled end fragments resulting from the enzyme digestion using a fluorimager or anti-fluorescein-AP antibody and thus determine the restriction maps.

PCR with 5-methyl-dCTP replacing dCTP generates fully methylated PCR products, which have been used as substrates for assaying the sensitivity of different restriction enzymes towards methylated DNA templates (1). Over a hundred restriction enzymes, such as Sau3A, HindIII, PstI and SacI, are known to be sensitive to fully or hemi-methylated restriction sites (2). Here, we attempted to generate partially-methylated PCR products by adding a mixture of 5-methyl-dCTP and dCTP. We demonstrated here that a partial restriction digestion pattern could be obtained by digesting the PCR products completely with methylation-sensitive restriction enzymes. This method eliminates the optimization steps needed for traditional partial digestion (3). The combined use of a dam methylase and MboI has previously been used to create partial digestion (4). However, to extend the method to use other combinations of a DNA methylase and a restriction endonuclease, careful and detailed studies are necessary to determine the ratio of the two enzymes to be used. Here, by controlling the ratio of 5-methyl-dCTP and dCTP in a PCR reaction, we can easily control the extent of methylation in the PCR products which are available for digestion with any 5-methyl-dCTP-sensitive enzyme to create a partial digestion.

An initial experiment was performed on a 2.2 kb DNA fragment cloned in pBluescript (Stratagene, La Jolla, CA). PCR products were obtained with the GeneAmp XL PCR Kit (Perkin-Elmer, Norwalk, CT, USA). In a total volume of 100 µl, 4 U of rTth DNA polymerase (Perkin-Elmer Corporation, Foster city, CA) was used in the recommended buffer with 0.5 µM primers (T7 primer, 5'-GTAATACGACTCACTATAGGGC-3' and T3 primer, 5'-AATTAAACCTCTACGAAAGGG-3'), 50 ng plasmid DNA template and 200 µM of each dNTP. To generate partially-methylated PCR products, 5-methyl-dCTP (Boehringer Mannheim Corp., Indianapolis, IN) was then added in addition to the unmodified dNTPs to final concentrations of 100, 150 and 200 µM as stated in the legend of Figure 1. A manual hot start was used to increase specificity by adding the rTth DNA polymerase to each individual reaction tube when the reaction buffer had reached 72°C. Furthermore, either one of the two primers was labeled with fluorescein (Genosys, Woodlands, TX) so fluorescein-labeled fragments could be detected (Fig. 1B) with a FluorImager SI (Molecular Dynamics, Sunnyvale, CA). The reactions were heated to 72°C for 10 min and followed by 30 cycles of 94°C for 30 sec, 45°C for 1 min and 72°C for 5 min. Then, each of the 100 µl PCR products were purified with a Centricon-100 (Ambion Inc., Beverly, MA) by washing with 2 ml sterile water twice and concentrated into 60 µl water for subsequent restriction digestion.

Purified PCR products (20–30 µl) were arbitrarily digested with 5 U of Sau3A (Stratagene, La Jolla, CA) at 37°C for 4 h to completion. The restriction digestions were analyzed with a 1.2% agarose gel (Fig. 1). Partial digestion patterns shown in lanes 3–5 and lanes 7–9 (Fig. 1A) indicated that Sau3A restriction sites of the PCR products were randomly protected from digestion when 5-methyl-dCTP was added to PCR reaction mixtures.

Analysis of the relative band intensities on lanes 3–5 or lanes 7–9 revealed two interesting properties of the partial digestion patterns. Firstly, the concentration of 5-methyl-dCTP from 100 to 200 µM did not affect the general restriction digestion pattern except for the largest band (the uncut, fully protected PCR product), which was more abundant at higher concentrations. Thus, a concentration of 100 µM of 5-methyl-dCTP should be

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Figure 1. Sau3A complete digestion of a 2.2 kb PCR fragment generated with different concentrations of 5-methyl-dCTP in the dNTP mixture. (A) Analysis of restriction fragments in a 1.2% agarose gel, stained with ethidium bromide and visualized in a FluorImager SI (Molecular Dynamics, Sunnyvale, CA) without a filter. Lanes 1–5, fluorescein-labeled T3 with regular T7 primers; Lanes 6–10, regular T3 primer with fluorescein-labeled T7 primers. Lanes 1 and 10, uncut PCR product; lanes 2 and 6, no 5-methyl-dCTP added; lanes 3 and 7, 100 µM 5-methyl-dCTP was added; lanes 4 and 8, 150 µM 5-methyl-dCTP was added; lanes 5 and 9, 200 µM 5-methyl-dCTP was added. M, HindIII-digested lambda DNA and HaeIII-digested φ174 DNA were used as size markers. DNA sizes in kb are shown to the left. (B) Same agarose gel but visualized with a band filter 530 ± 30 to detect signal from the fluorescein. An arrow indicated a minor non-specific PCR product. (C) Restriction map of Sau3A sites deduced from (B).

sufficient to generate a partial digestion pattern. Secondly, some of the bands are more abundant. These bands, which appeared darker in the partial digestion patterns, are likely to be fragments derived from PCR products which were only cut once by Sau3A. These fragments will then be tagged with either T3 or T7 primers. This argument was confirmed by detecting fluorescent signals generated from the fluorescein-labeled restriction fragments (Fig. 1B). These relatively abundant fragments were all tagged with either T3 or T7 primer at one of their ends. From the sizes of the fluorescein-labeled primer, a restriction map for Sau3A was deduced (Fig. 1C).

To extend the application to larger fragments, we have obtained partial digestions from 3, 4, 11 (data not shown) and 18 kb fragments (Figs 2 and 3). Unlike amplification with smaller fragments, the yield of the 18 kb PCR products decreased dramatically when the concentration of 5-methyl-dCTP was increased to 150 µM (Fig. 2, lane 4). Digestion of the 18 kb PCR products completely with HindIII produced partial restriction patterns (Fig. 3) with similar observations as described above. However, the fluorescent signals from the fragments labeled with fluorescein were very weak when detected directly with the FluorImager, which were probably due to the quenching effect of longer DNA fragment and less DNA molecules of larger fragments for the same amount of DNA. Thus, Southern blot and detection of fluorescein-labeled fragments with anti-fluorescein-AP antibodies was performed (Fig. 3B) as described previously (5). Restriction map was thus obtained (Fig. 3C).

To conclude, a novel method to achieve partial digestion from any DNA fragment that can be amplified by PCR has been developed. Our approach has eliminated the steps in traditional partial digestions which require the termination of restriction digestion reactions at various time points or the use of limiting restriction enzyme (3) or the use of specific cloning vectors (6). Adoption of fluorescein-labeled primers in this method allows one to determine the restriction map directly with a FluorImager if the fragment is about 3 kb or less. However, for longer
Figure 3. HindIII complete digestion of an 18 kb PCR fragment generated from a lambda DASHII clone as shown in Figure 2. Fluorescein-labeled T3 and regular T7 primers were used. (A) Analysis of restriction fragments in a 0.7% agarose gel, stained with ethidium bromide and visualized in a FluorImager SI (Molecular Dynamics, Sunnyvale, CA); lane 1, uncut PCR product; lane 2, no 5-methyl-dCTP added; lane 3, 50 µM 5-methyl-dCTP was added; lane 4, 100 µM 5-methyl-dCTP was added; lane 5, 150 µM 5-methyl-dCTP was added; M, HindIII-digested lambda DNA were used as size markers. DNA sizes in kb are shown to the left. (B) Same agarose gel but Southern blotted and fluorescein-labeled fragments were detected with anti-fluorescein-AP antibodies. Arrow indicated the detected fluorescein-labeled fragments. #, non-specific PCR fragments. (C) Restriction map of HindIII sites deduced from (B).

fragments, Southern blot and detection of fluorescein-labeled fragments will be necessary to obtain a restriction map. For other applications, we have successfully used the method to generate nested Sau3A deletion clones which are useful for gap closure in a sequencing project by cloning the Sau3A partially-digested PCR products into a BamHI and SmaI double digested cloning vector (manuscript in preparation). Currently, we are using the method as a DNA fingerprint to study phylogenetic relationships among several genetic loci in different subsurface bacterial strains. This method should also be applicable to any study that partial digestion is used such as the detection of polymorphisms of various known genetic loci in different organisms (7).

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