4922–4923 Nucleic Acids Research, 1995, Vol. 23, No. 23

Cytosine specific DNA sequencing with hydrogen peroxide

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Received October 31, 1995; Accepted October 31, 1995

We report a simple protocol for a C-specific chemical DNA sequencing reaction with hydrogen peroxide. Chemical sequencing offers unique advantages over dideoxy sequencing for specific applications, which include the study of DNA–protein interactions ('footprinting'), probing for alterations in DNA structure, and oligonucleotide sequencing. While dideoxy sequencing is often preferred over chemical methods, chemical sequencing can be useful for 'problem templates', for example regions with very high GC-content which have artifact bands in all four lanes due to 'polymerase stops'.

Compared with the original C-specific chemical sequencing reactions with hydrazine (1), the hydrogen peroxide reaction is attractive because it uses a less toxic, more stable, and more easily available reagent. When used in combination with other alternatives to the original base-specific chemical sequencing reactions that have been described in the literature (2–6), the entire chemical sequencing procedure can be based on chemicals of reduced toxicity, while simultaneously being more efficient and robust than the original Maxam–Gilbert protocol.

The base-specific modification of single-stranded DNA with 2–3 M hydrogen peroxide in Tris and carbonate buffers has been described (7). The reaction is specific for C-residues at pH 7–8.5, and T-specific if the pH is >9. Under these conditions, reaction with double-stranded DNA is much slower (7), and we could not obtain readable sequence patterns from double-stranded DNA. When triethyl ammonium acetate buffer was used instead of Tris, however, we observed C-specific modification of double-stranded DNA which yielded perfectly readable sequence patterns (Fig. 1).

Individual colonies from 20 different libraries in multiplex vectors were pooled and grown together in 40 ml SOA medium overnight (4). DNA was prepared using the Qiagen tip 100 columns according to the manufacturers instructions, digested with *Not*I, precipitated, and resuspended in 60 µl H_2O to a typical concentration of 0.3 µg/µl. Five microlitres of DNA was transferred to a V-bottom microtiter plate with hydrophilic coating ('AGTC plates'; Advanced Genetic Technologies, Gaithersburg, MD), and 15 µl of a freshly prepared H_2O_2 solution [245 mM H_2O_2 (Sigma), 10 mM EDTA, 133 mM triethyl amine acetate pH 7.0 (Perkin-Elmer)] was added. After taping the plate, vortexing for 5 s, brief centrifugation to collect solutions at the bottom, and incubation at room temperature for 20 min, the plates were frozen on dry ice for 10–15 min. After lyophilization overnight, 65 µl of 1 M piperidine were added, the plates were sealed with adhesive tape, and incubated in a 90°C oven between two preheated glass plates for 40 min. After cooling, 7 µl 3 M sodium acetate and 120 µl isopropanol were added to each well. The plates were taped, vortexed, and centrifuged at 4000 r.p.m. for 30 min. The supernatant was poured off, the plates blotted onto paper towels, and the pellet was washed twice with 150 µl 80% ethanol. After pouring off the second wash, plates were spun upside-down at 400 r.p.m. to remove the ethanol. Pellets were resuspended in 60 µl 0.3 M sodium acetate, and the ethanol precipitation and washing was repeated as described above. After a final upside-down spin at 400 r.p.m., the samples were resuspended in 10 µl formamide load mix (40% formamide, 0.05% bromophenol blue) and loaded onto direct transfer electrophoresis gels. Sequence patterns were visualized by hybridization with ^{32}P-labeled oligonucleotides and exposure to X-ray film (4).

The results shown in Figure 1 demonstrate the high specificity of the hydrogen peroxide reaction. The presence of both EDTA and triethyl ammonium acetate (TEAA) was critical to obtain C-specific patterns. When Tris–HCl, sodium borate, or sodium acetate buffers were used instead of TEAA, no readable sequence patterns could be obtained from double-stranded DNA. A wide range of hydrogen peroxide concentrations (<0.1–3 M) gave C-specific reactions. High concentrations (>1 M), however, often led to 'fuzzy' bands. Other apparent causes of blurry bands were insufficient vacuum during lyophilization, and too short lyophilization times.

During the past 5 years, we have used this reaction to generate >30 million bases of ('raw') sequence in a number of different multiplex sequencing projects. During this time, the protocol has undergone a number of minor variations. For example, piperidine removal for all four reactions shown in Figure 1 was originally done by lyophilization rather than by ethanol precipitation. However, we observed that higher quality patterns can be obtained with precipitation, and that sample loss during the precipitation is minimized when hydrophilically coated microtiter plates (AGTC plates) are used.

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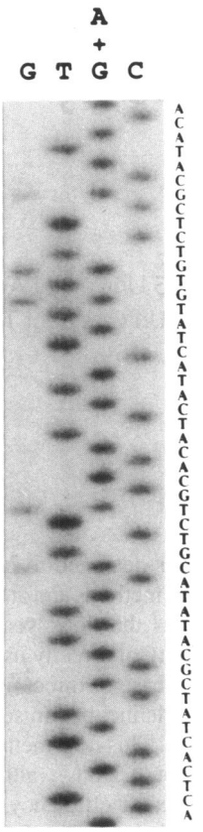

Figure 1. Sequence pattern obtained by chemical sequencing with hydrogen peroxide (lane C), as described in the text. The other lanes shown were produced by chemical sequencing with dimethyl sulfate (4) (lane G), potassium permanganate (5) (lane T), and acetic acid (4) (lane A+G), followed by cleavage with 1 M piperidine and ethanol precipitation as described in the text. The T-specific reaction was done according to the following variation of the protocol described by McCarthy (5). 5 µl of DNA were preheated to 90°C for 3 min in a thermal cycler (MJ Research, Watertown, MA). 10 µl of 0.1 mM $KMnO_4$ (freshly diluted in 2 mM sodium borate pH 9.5) were rapidly added using a multichannel pipettor, and the reaction was heated again for 80 s in the thermal cycler. The reaction was stopped immediately by adding 50 µl 1.3 M piperidine, and incubated for another 40 min at 90°C in the thermal cycler.

ACKNOWLEDGEMENTS

We thank Elena Vassilieva and Vladimir Shutov for translations of the Sverdlov and Kalinina paper into English. This work was supported by grants from the Department of Energy to GMC (DE-FG02-87ER60565) and by the National Center for Human Genome Research to JM (R01 HG000520).

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