Improvement of PCR sequencing by formamide

Wei Zhang, Guiying Hu and Albert Deisseroth*

The Department of Hematology, The University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, USA

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Direct sequencing of PCR products by T7 DNA polymerase is prone to problems in GC-rich regions, due to the stability of secondary structure and rapid reannealing of the heat-separated complementary strands of DNA.

To overcome these difficulties, we examined the effect of adding formamide to the sequencing reaction. Formamide weakens the hydrogen bonding between nucleotides (1, 2), and is, therefore, of great advantage in the sequencing of PCR products of p53 gene, which contains two GC-rich (62%) regions. The sequencing ladders of the two GC-rich regions of p53 obtained in the presence of increasing percentages of formamide is shown in Figure 1. In both cases, inclusion of formamide dramatically increased the intensity of specific bands, dissolved several local secondary structures, and eliminated the presence of most bands which crossed over all four lanes, especially at the regions close to the annealing of the sequencing primers (Figures 1A and 1B, arrowheads). The percentage of formamide required to generate the improvement of sequencing depends on the percentage of GC nucleotides in the target sequence. It is, therefore, often necessary to empirically determine the amount of formamide needed to resolve secondary structures completely. A useful formula can be used for determination of the amount of formamide needed: $\text{Formamide\%} = 0.7 (\text{GC\%} - 50)$. With this percentage of formamide, the T_m of the DNA duplex is equivalent to that of DNA duplex with 50% GC nucleotides (1, 2). We also noticed that some nonspecific bands of secondary structure were not totally abolished (Figure 1A, arrow), even when we increased the percentage of formamide to 30% (data not shown). Under these conditions, the signal intensity of the sequencing ladder became weaker due to the decreased efficiency of template to primer binding. The insensitivity of T7 DNA polymerase to formamide is not unusual. Formamide has also been shown to improve the specific amplification of GC rich sequence in PCR reaction driven by Taq DNA polymerase (3).

We conclude that formamide provides an easy and effective means with which to extend the capability of PCR sequencing for the generation of important sequence information in genes of high GC content.

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Figure 1. 0.2 μ g PCR products of p53 gene were mixed with a five molar excess of sequencing primers in Sequenase buffer with the increasing percentages of formamide indicated in each lane. The annealing mixture was boiled for ten minutes and quickly cooled on dry ice ethanol bath. Sequencing reactions using the USB Sequenase kit, in which the percentage of formamide decreases to 62% of that in the annealing reaction, were carried out on ice for ten minutes, then aliquoted to prewarmed ddNTP, and reacted for ten minutes at 42°C. The reaction was stopped by adding stop solution. The sequencing reactions were heated to 95°C for 3 minutes before loading. The loading pattern was GATC. The sequencing gel was run at 50W for two hours, fixed in 15% methanol, 5% acetic acid, dried, and exposed to X-AR film for two–three days. 35 S-dATP was used in the sequencing reaction. 1A and 1B represent two different GC-rich regions of p53 gene.

*To whom correspondence should be addressed at Department of Hematology, Box 24, M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA