Imidazole Improves Specificity of PCR by Suppressing the Product-Priming Outgrowth

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Imidazole eliminates the multiple sub-bands when random pool DNA is used as template for PCR. It is suggested that imidazole as a denaturant improves the specificity by suppressing the sub-bands outgrowth using the formerly amplified template as primer.

The polymerase chain reaction (PCR) is one of the most indispensable tools in *in vitro* selection or evolutionary molecular engineering.[1] Because of the powerful amplification potential of this technique, undesirable multiple satellite bands, in addition to the single expected band are often detected by denaturant polyacrylamide gel electrophoresis. These multiple bands, which are frequently attributed to non-specific priming, often very difficult to eliminate even when all the experimental parameters are optimized. In this case, the use of denaturants has been reported to improve the specificity of PCR.[2, 3]

We have found that the inclusion of imidazole in the PCR mixture could dramatically reduce and even eliminate these multiple sub-bands, when random pool ssDNA (100-mer with 60-nucleotide random sequence region flanked by 20-nucleotide fixed regions at both the 5′and 3′ends) was used as template for PCR using 5′- and 3′- primers containing BamHI and HindIII restriction enzymes sites, respectively (Lane 3, 4, 5, 6 and 7 in Figure 1). Titration study showed that imidazole at a concentration of 75-150 mM (1 M=1 mol dm $^{-3}$) can effectively eliminate undesirable amplification. The same results were obtained when histidine or formamide was added into the PCR solution (Lane 8 and 9 in Figure 1). Using other templates, we have found that imidazole can effectively eliminate only the sub-band longer than the template and not the ones shorter than the template. The same results were obtained from PCR with annealing temperature between 64-68 °C, and from PCR with 1.5-3 mM $MgCl_2$. It was also shown that the upper multiple sub-bands emerged only after 20 cycles of PCR without imidazole.

Imidazole is known to have a nucleophilic functional group that can directly participate in the catalytic process such as hydrolysis of RNA.[4] In contrast to RNA, no such effect is observed for DNA. But, imidazole induces destabilization of DNA double helix. We observed that 1M imidazole could decrease the melting temperature of the double helix of the above-mentioned DNA by 10 ± 2 °C.[5]

In order to elucidate the nature of the upper multiple sub-bands that can be eliminated with imidazole, we performed the following investigations. Restriction fragments length analysis with BamHI and HindIII suggested that both the restriction sites were included in the DNA of the sub-bands, and that the distribution of the two kinds of restriction sites was a tandem repeat of that in the template. Temperature gradient gel electrophoresis (TGGE) [6, 7] of the products of PCR without imidazole gave additional two clues (Figure 2). First, the chain separation temperature of double-stranded DNA from both the main and sub-band is almost same. Second, in the case of the main band, the mobility of the double-stranded state is greater than the single-stranded random coil state. On the other hand, in the case of the sub-band, the mobility of double-stranded state is smaller than the single-stranded random coil state. As the final cycle of PCR was stopped at the extension temperature, the double-stranded state of main band is fully helical duplex and gave normal mobility transition at the chain separation temperature. The mobility transition pattern for the sub-band, however, is a typical one for the case of double-stranded DNA having a big internal loop clamped by a weak clamp and a strong clamp. In our case, the following model is plausible. The first half of the double-stranded DNA is fully helical duplex, and the other half has a big internal loop composed of random 60-nucleotide region clamped by primer binding sites at both ends. This model implies that the major sub-band is composed of tandem duplicated template and was amplified by the use of the formerly amplified template and its complementary strand as primers. The possibility of simple miss-priming or self-priming for the origin of the major sub-band is ruled out by the above-mentioned data of restriction fragments.

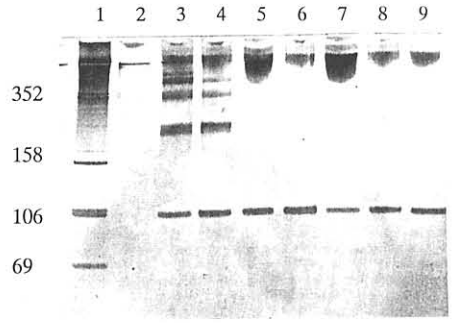


Figure 1. Specificity of PCR amplification of random pool ssDNA in response to various additives. Random ssDNA pool of diversity 10^{10} (100 fmol) was amplified in 100 μl PCR buffer (50 mM Tris-HCl, pH 8.8; 15 mM $(NH_4)_2SO_4$; 2.5 mM $MgCl_2$; 0.45 % Triton X-100 ; 200 μg / ml bovine serum albumin) including 10 pmol of each primers (5′ GTC**GGATCC**TAGCTCCACAT, biotin---5′ GGCTC**AAGCTT**AGCGAACCA), 200 μM of dATP, dTTP, dGTP, and dCTP, 1unit Taq DNA polymerase (Biotech International) and denaturant. The mixture was cycled 30 times on a thermal cycler (MJ Research Inc.) after it was treated at 95 °C for 180 s. The temperature cycle was 94 °C for 30 s, 66 °C for 30 s and 72 °C for 30 s. Lane 1: HaeIII-treated fd DNA size marker; Lane 2: product of PCR without DNA; Lane 3: products of PCR of random pool ssDNA without any additives; Lane 4,5,6 and 7: products of PCR of random pool ssDNA with 50 mM, 75 mM, 100 mM, and 150 mM imidazole, respectively; Lane 8 and 9: products of PCR with 100 mM histidine and 2% formamide, respectively. Electrophoresis was performed at 65 °C in 6% (w/v) polyacrylamide gel which included 8 M urea.

The effective elimination of such a product-priming by using

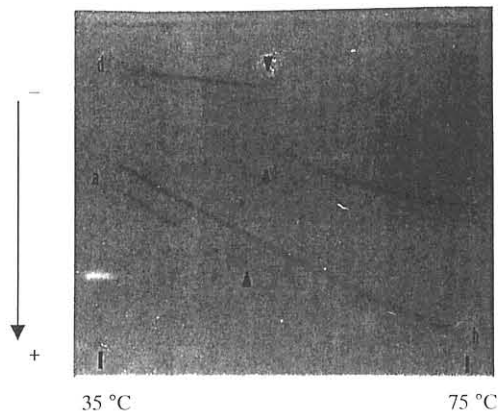
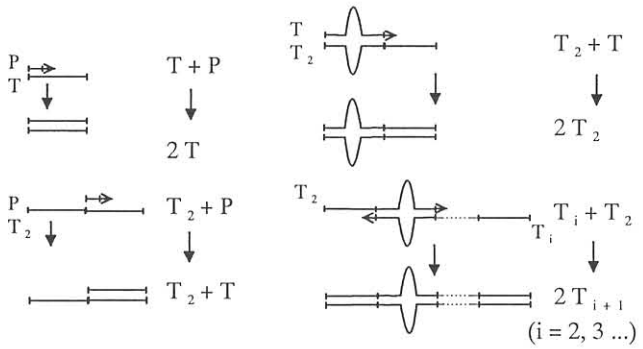


Figure 2. Perpendicular temperature gradient gel electrophoresis showing the mobility transitions of DNA. a: The double-stranded state of the main band. b: The single-stranded state of the main band. c: The single-stranded state of the sub-band. d: The double-stranded state of the sub-band. The arrowheads indicate the chain separation point of double strands. The temperature gradient was from 35 °C to 75 °C. The gel was run at 400 V for 3 h. The content of gel is same as Figure 1.

denaturant such as imidazole can be explained as follows. The amplification reaction of the multiple sub-bands(T_2, T_3, T_4,...) is described by



where P, T, and T_i denote primer, template and tandem-i-mer of the template, respectively, and the complementary strand is denoted by the identical symbol for simplicity. Up to the *ca.* 20-th cycle, P is in excess in amount of T or T_2, so that the left-hand side reactions dominate. After the *ca.* 20-th cycle, T becomes comparable in amount to P and therefore T_2 begins to be amplified by the third reaction. After accumulation of T_2, T_i ($i \geq 3$) begins to be amplified by the fourth reaction. Thus the denaturation effect of imidazole on the amplification of T_2 acts two times. This double action on the cascade reaction slows down the sub-bands growth more than main band growth and the sub-bands are apparently eliminated. This means that when PCR is performed in reduced number of thermocycle the sub- bands are also apparently eliminated without imidazole.

Possibility that imidazole suppresses only the first appearance of T_2 was ruled out by the experiment as follows. We extracted T_2 from the gel, then add it by the amount ten times less than T to the PCR solution with and without imidazole. The electrophoretic pattern showed elimination of the sub-bands with imidazole.

The mechanism of the first appearance of T_2 is under investigation.

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