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Thermal profile with alternately raised and lowered annealing temperature improves the PCR amplification using highly degenerate primers[o]

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The proposed here PCR thermal profile improves the specificity and efficiency of PCR using highly degenerate primers, especially in the case of larger PCR products (around 2000 bp and more). The improvement is achieved by the use of a specific annealing temperature in the beginning cycles and the alternate lowering and raising of the annealing temperature in the subsequent cycles.

PCR using degenerated primers presents a very attractive way of obtaining genes of unknown nucleotide sequences [1]. The more degenerated primers, the better chance for complementary primer-matrix matching occurs, but the specificity and efficiency of PCR amplification are usually lowered. This is because a very small fraction of primers is fully complementary to the martix sites and only a small part of primer fractions is utilised, whereas the rest is either useless or contributes to non-specific amplifications. However, primers with mismatches could be also useful for amplification provided the annealing temperature little lowered and the mismatch is not situated exactly at the 3′ end of a primer. Unfortunately, the lowering of annealing temperature usually results in formation of non-specific products. These non-specific small products are amplified usually much more efficiently than the large, specific ones, and in consequence, inhibits competitively formation of the large product. Thus the large, desired product may be even invisible on ethidium bromide stained gels. Increasing primer concentration without the lowering of annealing temperature is helpful, but only to some extent, and in the case of highly degenerate primers (e.g. 4096-fold) seems to be insufficient. The standard PCR mixture (50 μl vol-

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ume, 1 μM primer concentration) comprises 50 pM each primer. The formation of 1 μg of 2000 bp PCR product requires incorporation of 0.75 pM of each primer. In the case of 4096-fold degenerate primer the amount of fully complementary fraction could be only around 0.0125 pM and 0.125 pM upon a tenfold increase of its concentration, so about six times less than necessary to obtain 1 μg of DNA, in 50 μl reaction volume. This gives a band, well visible on agarose gels if 10 μl of such a mixture is loaded per lane. In practice even at the specific annealing temperature some mismatched primers may work, but, on the other hand, an efficient reaction requires an excess of substrates. Obviously, there are more sensitive PCR product detection methods than ethidium bromide staining of agarose gels, but cloning procedures require rather microgram amounts of DNA, especially if purification or DNA band isolation are necessary, to obtain unique strong DNA bands on agarose gels.

Taking all these data into consideration we claim that the utilisation of mismatched primers in the PCR reaction is necessary, but the decrease of annealing temperature leading to non-specific product formation is not desired. Since such a decrease cannot be avoided, it may be delayed by gradual lowering of the annealing temperature. Furthermore we can take advantage of the fact that one strand of the PCR product with incorporated mismatched primers is also a PCR matrix for a fraction of mismatched primers. If the mismatch leads to transition of A or T into G or C, the annealing temperature may be higher for the primer complementary to such a matrix. For example, let us consider a model where the specific starting temperature is 50°C. Assuming that after some cycles the fraction of the fully complementary primers is exhausted, we may lower the annealing temperature to 49°C so as to utilise the primers with one mismatch. The amount of primer with one mismatch is the multiple of the amount without mismatch. We could wait until the fraction of one mismatch primers is exhausted and then lower the annealing temperature to 48°C to utilise the primers with two mismatches in relation to genomic DNA matrix, but with one mismatch in relation to the previously formed PCR products. However, if we increase the annealing temperature to 50°C again no PCR product forms on genomic DNA matrix because the fraction of fully complementary primers is exhausted, however, the PCR products may be synthesised on the matrix of the PCR products containing A or T instead of G or C introduced by primers. Thus we may alternately decrease and increase the annealing temperature. As the decrease of annealing temperature would be delayed, the formation of non-specific products could start not earlier than in the final cycles, which means that non-specific products would not be visible on agarose gels.

RESULTS AND DISCUSSION

The presented PCR thermal profile was based on the above described considerations and tested empirically in PCR amplification of glucosamine-6-phosphate synthase gene (2151 bp) from *Saccharomyces cerevisiae* genomic material. Obviously, we do not claim that the changes of annealing temperature took place precisely at the moment when a primer fraction became exhausted, but the general idea of the model accounts for the improved efficiency and specificity in the PCR experiment.

The PCR reaction mixtures contained in 50 μl: 5 μl of 10× reaction buffer (100 mM Tris/HCl, pH 8.8, at 25°C, 15 mM $MgCl_2$, 500 mM KCl, 1% Triton X-100), 5 μl of dNTP mixture (2 mM of each deoxynucleotide), 2 μl of gsu2 primer (TTA YTC NAC NGT NAC NGA YTT NGC, 4096-fold degenerated, 100 μM), 5 μl of gsu1 (ATG TGY GGN ATH TTY GGN TAY 3' 384-fold degenerated, 10 μM), 2 μl matrix DNA (0.1 μg/l), and 2 units of thermostable DNA polymerase Shark2 (DNA-Gdańsk, Poland). Amplifications were carried out on a

Biometra Personal cycler (Germany). After amplification 10 μl samples were subjected to electrophoresis on standard 1% agarose gel stained with ethidium bromide. The annealing temperatures were calculated according to the following formula:

$$T_m = 81.5 + 16.6\ (\log_{10} \times [J^+]) + 0.41[\%(G + C)] - (600/l),$$

where: $[J^+]$ = cation concentration in moles (KCl, $MgCl_2$, dNTPs) = 0.06 M, l = oligonucleotide length (number of bases), [%(G + C)] = G,C content.

Figure 1 presents four thermal profiles and results of PCR amplifications.

The idea of profile I is based on the assumption that matching of the primers with non-complementary bases to genomic DNA matrix requires long annealing at low temperature in a few beginning cycles. Then the annealing temperature is raised. Such a thermal profile [2] gave positive results in many cases but it was inefficient in our case. The desired, specific product is almost invisible, the smaller non-specific products are prevailing (Fig. 1, lane I). This strongly suggests that the specific products could be synthesised efficiently because the non-specific products were formed in large amounts, in the beginning cycles and, as they are much smaller, were synthesised much more efficiently. It seems that this type of thermal profile may be more appropriate for amplification of smaller products, using less degenerate primers (i.e. less than a few hundredfold or a few thousandfold like in this case).

In profile II the annealing temperature is 48°C. It is the calculated temperature for the primer fraction with the highest AT content. Similarly as in profile I, the desired specific product is almost invisible and the smaller non-specific products are prevailing (Fig. 1, lane II).

In profile III the annealing temperature is 52°C. It is the calculated temperature for the

A

Thermal profile I

93°C, 2 min	
93°C, 30 s	}
36°C, 2 min	}6 cycles
72°C, 2 min	}
93°C, 30 s	}
52°C, 1 min	}36 cycles
72°C, 2 min	}
72°C, 2 min	

Thermal profile II

93°C, 2 min	
93°C, 30 s	}
48°C, 1 min	}42 cycles
72°C, 2 min	}
72°C, 2 min	

Thermal profile III

93°C, 2 min	
93°C, 30 s	}
52°C, 1 min	}42 cycles
72°C, 2 min	}
72°C, 2 min	

Thermal profile IV

93°C, 2 min	
93°C, 30 s	}
52°C, 1 min	}6 cycles
72°C, 2 min	}
93°C, 30 s	}
51°C, 1 min	}6 cycles
72°C, 2 min	}
93°C, 30 s	}
50°C, 1 min	}6 cycles
72°C, 2 min	}
93°C, 30 s	}
49°C, 1 min	}4 cycles
72°C, 2 min	}
93°C, 30 s	}
50°C, 1 min	}3 cycles
72°C, 2 min	}
93°C, 30 s	}
49°C, 1 min	}3 cycles
72°C, 2 min	}
93°C, 30 s	}
48°C, 1 min	}4 cycles
72°C, 2 min	}
93°C, 30 s	}
49°C, 1 min	}3 cycles
72°C, 2 min	}
93°C, 30 s	}
48°C, 1 min	}7 cycles
72°C, 2 min	}
72°C, 2 min	

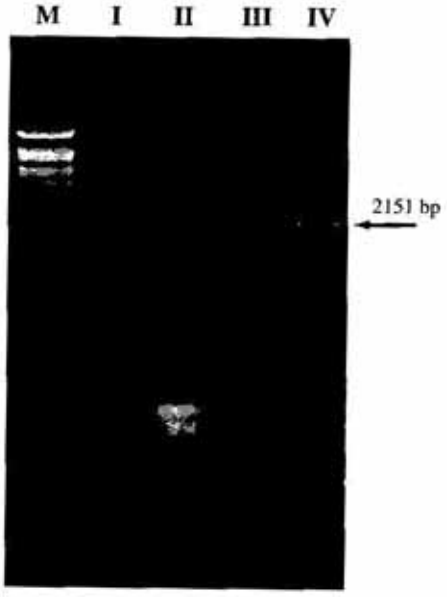

Figure 1. Four PCR thermal profiles and the results of amplifications.

A. Amplification profile using the indicated temperature regiones. For details see the text. M, DNA relative molecular mass standard λ/*Bst*EII (digestion fragments: 8454, 7242, 6369, 5686, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702, 224 (non visible), 117 (non visible)).

primer fractions with the highest GC content. No amplification products are visible (Fig. 1, lane III).

In profile IV the annealing temperature has been changed several times in the range of 52–48°C. The expected product is formed here efficiently (Fig. 1, lane IV). The specificity of the amplification is satisfactory, only one, small non-specific product is formed. The identity of the PCR product of around 2200 bp was additionally confirmed by *Hin*dIII digestion giving the predicted restriction fragments of 700 bp and 1500 bp. It seems that this type of thermal profile allows to utilise more primers with bases non-complementary to the genomic DNA matrix. In the beginning cycles the complementary primers become utilized. Then, after they are exhausted, the lowered annealing temperature enables less complementary primers to be utilised. The raising of annealing temperature for a few cycles after it has been lowered for a few cycles is thought to delay the decrease of annealing temperature and thus to prevent the formation of non-specific products. We expect that after the PCR products with G or C introduced by primers instead of A or T, are formed they serve as the target for the primer fractions that could not be utilised before. As the GC content of the primer and the primer site on the matrix DNA becomes higher, the annealing temperature also may be raised. In consequence, the formation of non-specific products is reduced.

The amplification using thermal profile IV allowed to obtain PCR products of predicted size (over 2000 bp) also for the *Candida dubliniensis* and *Candida glabrata* matrices (not shown). The sequences of glucosamine-6-phosphate gene for those two organisms are unknown.

We think that the proposed type of the PCR thermal profile may be helpful in some cases in which degenerate primers are used. We suppose that this type of thermal profile is more suitable for highly degenerate primers (a few hundred, a few thousandfold) and bigger PCR products (about 2000 bp and more). As the proposed thermal profile is rather inconvenient in programming we recommend its application only when it is really necessary. Similar effects may be obtained also by gradual decrease of the annealing temperature every cycle (touchdown) or every a few (several) cycles, without alternate decrease and increase of the annealing temperature like in this study. However, it seems that the PCR thermal profile of the type proposed here should be more efficient.

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