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Low concentrations of tetramethylammonium chloride increase yield and specificity of PCR

Eric Chevet, Gilles Lemaître and Michaël Doron Katinka*

Laboratoire de Recherche sur la Croissance Cellulaire, la Réparation et la Régénération Tissulaires, URA No. 1813 CNRS, Université Paris XII – Val de Marne, 61, Avenue du Général de Gaulle, 94010 Creteil Cedex, France

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Polymerase chain reaction (PCR) and its diverse technical extensions and developments has evolved into the method-of-choice to isolate and amplify specific segments of DNA out of a complex mixture of sequences (1). In PCR, two oligonucleotides serve as reciprocal primers flanking the DNA fragment matrix to be amplified. Some simple rules help design efficient primers. Ideally, primers should be 16–26 nucleotides in length, contain 50–60% guanines and cytosines (GC) and anneal to the matrix at ~55°C (0–10°C below the T_m: temperature at which 50% of the DNA molecules are denatured; 2). The numerous protocols designed for the different PCR applications almost always require an optimisation step when put into practice. We frequently undertook PCR experiments as part of our studies on the adenine and thymine (AT)-rich macronuclear genome of *Paramecium primaurelia* (80% AT content; 3). The low annealing temperatures required often induced misannealing, resulting in unacceptably high non-specific amplification of the DNA (not shown). To overcome this problem, we studied the possibility to increase the T_m by the addition of tetramethylammonium chloride (TMAC; Merck, Schuchardt, Germany) to the reaction mixture, thus improving specific annealing of the primers. At 3 M TMAC an AT base pair (bp) is thermally as stable as a GC base pair (4), the overall stability depending on the length of the annealing sequences only. At such concentrations however, TMAC is usable in Southern membrane hybridization experiments (5) while it completely inhibits DNA polymerase activity (Fig. 1B and results not shown).

In this work we studied the influence of low concentrations of TMAC in PCR with primers of various GC contents and a 15 742 bp matrix coding the 156G surface protein gene of *P.primaurelia* inserted in pUC19 (6). As an illustration, pairs of PCR primers were selected among the following five 17mer oligonucleotides (Genosis, Cambridge, UK) (see Fig. 1A): **1.** CGCTGCTTGTGCTTGGG (T_m = 67°C, 59°C, the T_m differs if calculated according to reference 7 or to reference 8, respectively); **2.** TCGACACAGGCAGTTCC (T_m = 61°C, 54°C); **3.** ACATGTAGTTCTAGTTA (T_m = 37°C, 45°C); **4.** ATGCACATGTGCATAAT (T_m = 52°C, 47°C) and **5.** ATTAAAAACAAATAATA (T_m = 38°C, 38°C). The expected sizes of the amplified DNA fragments are: 593 bp with primers 1 + 2 (p1 + p2), 711 bp with p3 + p4 and 947 bp with p3 + p5. PCR was carried out with 10 pg plasmid DNA, 1 µg yeast tRNA (Life Technologies, Gaithsburg MD), 0.1 µM of each primer, 10 mM KCl, 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mM of each dNTP (Pharmacia, Uppsala, Sweden) and 1 U Vent polymerase (New England BioLabs, Beverly, MA) in a final volume of 50 µl, using the Thermojet thermal cycler (Eurogentec, Seraing, Belgium). PCR was carried for 20 cycles with 1 min denaturation at 95°C, 1 min annealing at 59°C, 46°C and 37°C with p1 + p2, p3 + p4 and p3 + p5, respectively, followed by 1 min extention at 74°C. Cycling was terminated by 10 min incubation at 74°C. PCR was carried out for 20 cycles to assure amplification under exponential phase conditions (not shown). A preliminary PCR experiment was carried out in the presence of increasing concentrations of TMAC. An example of the effect of TMAC concentration is shown in Figure 1B utilising p3 + p4. At 15 mM or more we observed an increase in the PCR yield, culminating at 60 mM. Over 150 mM, TMAC completely inhibited the reaction. The results with p1 + p2 and p3 + p5 were similar. The optimal TMAC

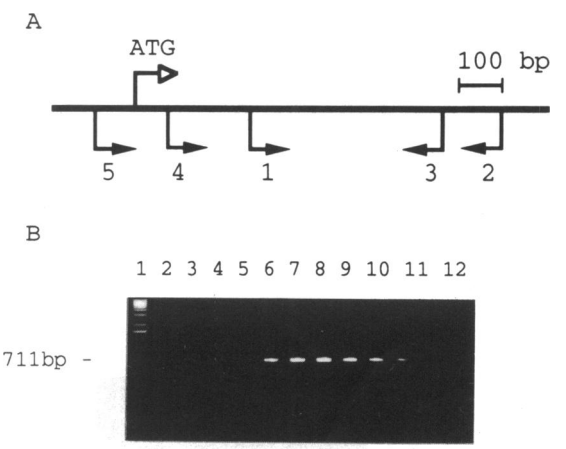

Figure 1. (**A**) Map including the localisation of PCR primers on *P.primaurelia* 156G surface protein coding DNA fragment. (**B**) PCR products using primers 3 and 4 (711 bp). Lane 1, molecular weight markers (0.5 µg 1 kb DNA ladder; Life Technologies, Gaithburg, MA). Lanes 2–12, one-tenth of the total PCR products obtained in the presence of 0, 1, 5, 10, 15, 30, 60, 90, 120, 150 and 200 mM of TMAC, respectively.

* To whom correspondence should be addressed

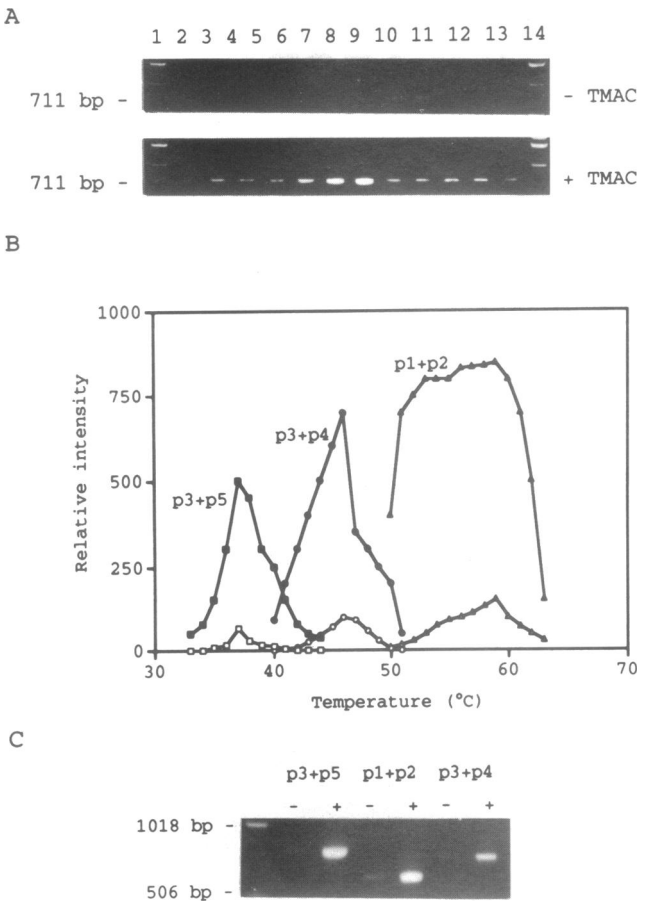

Figure 2. (**A**) PCR amplification products between primers 3 and 4 in the presence (+) or absence (–) of 60 mM TMAC as a function of the annealing temperature. Lanes 1 and 14, 0.5 µg of 1 kb DNA ladder (Life Sciences, Gaithsburg MD). Lanes 2–13, PCR products obtained at 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 and 51°C. (**B**) Representation of the relative intensity of the PCR products determined by densitometric analysis as a function of the annealing temperature, using the pairs of primers p1 + p2 (▲), p3 + p4 (●) and p3 + p5 (■) in the absence (open symbols) or in the presence (closed symbols) of 60 mM TMAC. (**C**) Products of *P.primaurelia* genomic DNA amplification in the presence (+) or in the absence (–) of 60 mM TMAC with primers p3 + p5 (947 bp in size), p1 + p2 (593 bp in size) and p3 + p4 (711 bp in size).

concentration (OTC) was determined to be of 60 mM (Fig. 1B and results not shown). In all the following experiments the OTC was used. We compared amplification both with or without TMAC as a function of the annealing temperature. As illustrated in Figure 2A and C (and results not shown), with any pair of primers used, TMAC not only enhanced amplification yield ~5–10-fold, but, it also greatly diminished non-specific priming. Unexpectedly, these effects of TMAC were observed for both low GC content primers p3 + p5 and for high GC content primers p1 + p2. The optimum annealing temperature for each pair of primers was not significantly different in the presence or the absence of TMAC. Quantification of the results obtained with p1 + p2, p3 + p4 and p3 + p5 are shown in Figure 2B. Maximum amplification was observed at 37°C (p3 + p5), 46°C (p3 + p4) and 59°C (p1 + p2).

To study the TMAC effect in PCR on DNA of higher molecular complexity, we undertook experiments with *P.primaurelia* macronuclear genomic DNA (~10^8 bp complexity of an haploid macronuclear genome; 9). DNA (2 ng) was amplified for 25 cycles at OTC and at the optimal temperature for each pair of primers. As shown in Figure 2C, results are very similar to those previously obtained with plasmid DNA, notably a 5–10-fold better yield and a reduction in non-specific priming.

At 3 M TMAC, the thermal stability of an AT bp is identical to that of a GC base pair. At the selected TMAC concentrations (60 mM), the slight increase of the AT bp stability as well as the exponential rate in which PCR progresses may explain the better specificity and yield of the PCR reaction products with either AT- or GC-rich oligonucleotides. A direct effect of TMAC on the catalytic properties of the DNA polymerase can also be suggested. In the present work we used the Vent DNA polymerase, an enzyme isolated from archaea *Thermococcus litoralia*. To study the TMAC effect in PCR on other thermo-resistant DNA polymerases TMAC will give the same results. We repeated the experiments at optimal temperature and OTC with *Taq* DNA polymerase *(Thermus aquaticus)* from Promega and Goldstar DNA polymerase *(Thermus* new strain) from Eurogentec. Results were similar with all the polymerases tested (not shown). Different ions (KCl, ammonium chloride, etc.) and solvents (ethanol, formamide, etc.) were tried out to reduce the T_m of primers used or to stimulate the DNA polymerase activity (2). In many cases the effect of these compounds was sequence dependent. In comparison TMAC offers both an enhancement in the yield of the PCR products and an increase of their specificity, while being sequence independent. Since the technique was developed, TMAC was used with satisfactory results in PCR experiments on bovine, mouse or rat DNA with various oligonucleotides.

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